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EDITED BY

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Preface

In common with many other scientific subjects, that of nutrition becomes more and more difficult to envisage as a whole because of the avalanche of published papers which envelops every worker in this field Even in specialized aspects of nutrition it is often difficult to keep pace with the course of events and the spate of publications The role of the review journal becomes thus ever more important in the comprehension of nutritional progress There are already in existence a number of review journals in America the United Kingdom, and Europe which fulfil a valuable role in interpreting the nutritional field, but I feel, together with the Advisory Editors, that there is more than ample scope for this new World Review We hope to provide a forum for more lengthy and discursive reviews than are published in conventional journals and we hope that these reviews will not be a catalogue of the papers published in the last few years Each one will be written by an expert in the particular field which is to be covered and should provide a critical evaluation of the field We hope it will bear the imprint of the author himself, that is to say his interpretation, his views and his theorizing will be welcomed as an integral part of his article

It is aimed to produce this review annually and to make it truly International In the present volume the following nationalities are represented United Kingdom, United States France Japan and Guatemala

These then are the aims and objects of the new review and it is to be hoped that our readers will find that the volumes will live up to them

It has been a great pleasure and an honour to have as authors in this first volume Prof E V McCollum and Prof C G King, who have carved for themselves a permanent place in the history of nutrition and the editor and publishers are greatly indebted to them for adding lustre to the new series We also owe a great debt of gratitude to our other very distinguished contributors for their confidence in us and in the new *World Review* by lending their abilities and their pens to Volume I

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12th January 1959

PUBLISHER S NOTE

IT is regretted that this volume has been seriously delayed by circumstances over which we had no control We wish to apologize for this delay and to seek the reader s indulgence if he is surprised at the omission in certain articles of references to important recent publications

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The History of Nutrition

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I INTRODUCTION

The *World Review of Nutrition and Dietetics* in its successive volumes will be written by people with special and expert knowledge of nutrition problems The objective is to describe the nutritional status of peoples under different geographic climatic and economic conditions and to examine all available knowledge in order to find solutions wherever nutrition problems exist The Editor invited me to introduce this new series of nutritional reviews by writing this chapter on the history of nutrition I shall endeavour to do this by giving an account of the more important events in thought and experiment by which the science of nutrition developed In the space available the story of progress in this field must of necessity be considerably abbreviated Elsewhere (McCollum 1957*a*)[1] I have given a detailed account of experimenters who contributed some hundreds of observations of importance mostly chemical which stimulated reflective minds and invited further inquiry and brought to light important new facts

The early writers on materia medica in describing medicinal plants employed such terms as farinaceous mucilaginous saccharine acidulous oily, caseous *gelatinous albuminous and fibrinous principles* Early chemists recognized only these properties in vegetable substances The pioneer physiologists wondered that foods so unlike each other and unlike any body tissues could be so metamorphosed in digestion as to form the various body structures in growth repair of injuries and for conversion into wool eggs milk etc Chemists had no explanation to offer The problem of what was important in foods and what unimportant was so perplexing that few were motivated to do more than speculate Nobody knew how to conduct experiments which offered any prospect of important discoveries

II PRIMITIVE MAN'S IDEAS ABOUT FOODS

Primitive peoples held very definite ideas about quality in foods Frazer (1931)[2] in his vast studies of ancient cults and folklore describes many food taboos Most of these had to do with beliefs that certain foods had magical powers Perhaps the most common idea was that the eater acquired the qualities of the animal whose flesh he ate either strength or weakness depending on the strength and courage or the timidity of the creature Modern experience in general has not confirmed these old beliefs

Primitive beliefs in the medicinal value of many plant substances contain the germ of the idea that they were *composed of different principles each one* effective in curing a different disease Such ideas were based either on observations or on authority of powerful persons It was not possible to verify or refute such claims until the advent of the science of chemistry It is worth our attention to consider what the most outstanding early philosophers thought about the nature of the physical world and especially of the origin of the organic matter of plants and animals

Thales of Miletus (650–580 B C) believed that water was the substance from which through condensation a primordial mud was formed and from this plants and animals were derived More than two thousand years later van Helmont believed that he had proved experimentally that in the growth of a willow tree 164 lb of wood had been formed from water Even in 1791 Dr George Fordyce[3] a prominent physician of London believed that experiments which he entrusted to an assistant proved conclusively that small minnows grew to much larger fish with only water and air available to them He did not suspect that his assistant secretly fed the fish

Fordyce was the first man to conduct a nutrition experiment using controls for comparison He noted that his canary hens looked poorly nourished as the egg laying season progressed He reasoned that the hens might need more calcareous substance for making egg shells than the ordinary diet of canary *seeds provided He* put the question to test He divided his canary hens into two parties to one of which he gave a piece of old mortar The birds ate it freely The results showed that the hens which ate of the mortar remained in good condition through the laying season whereas those which ate seeds only were malnourished and some died

In 1799 Vauquelin[4] one of the most *eminent* chemists of his time believed that he had demonstrated by experiment with a farmyard hen that transmutation of inorganic elements occurred in the metabolic processes The question whether in the process of vegetation plants generated or transmuted inorganic substances was unsettled until in 1842 Wiegmann and Pollstorff[5] proved conclusively that all mineral substances *in plants* were derived *from* the soil

III EARLIEST CONCEPTIONS ABOUT FOODS

Reasoning about why herbs possessed medicinal properties was impossible so long as philosophers believed that water, air fire and earth were the primordial materials from which all things even the human body (Empedocles) were formed by uniting them in different proportions and that health and sickness depended on the harmony or disharmony of the four humours blood phlegm black bile and yellow bile (Hippocrates)

Paracelsus (1493-1541) introduced the concept of the body as a chemical system He accepted the idea of the four elements but assumed the presence in the body of a mysterious force, which he called *archaeus* and which he conceived to be endowed with the power of dominating the life processes Health and disease depended upon the mood of the *archaeus* Van Helmont (1577-1644) the discoverer of carbonic acid gas accepted the views of Paracelsus

The actual founder of iatrochemistry (medical chemistry) was F de le Boe Sylvius (1614-1672)[6] who assumed that processes of physiology and pathology resulted from what he called fermentation He attributed *metamorphosis* of food to the action of saliva and a ferment secreted by the pancreas and believed that peculiar virtues of the blood derived from ferments contributed to it by the bile and the lymph glands If ferments were in right proportions and amounts the body was in a state of health Disease was caused by an acrimony a superabundance of acid or alkaline substances generally or locally

The philosophy of Sylvius like that of Paracelsus was inadequate to a comprehension of the properties of foods and of the processes of nutrition But the view that many plants were endowed with medicinal properties suggested the idea that in the disordered states which their administration corrected something that was missing in the body was supplied by the remedy The hundreds of remedies described in early pharmaceutical writings suggested complexity of body chemistry but could not illuminate the relations between food and nutrition

1 Hilaire M Rouelle s Design of a System of Chemical Analysis

H M Rouelle (1718-1778)[7] an apothecary in Paris about the middle of the eighteenth century suggested that the best way to dissect plant materials was to apply to them successively a series of organic solvents the poorest one to be applied first the next poorest second and so on By this technique specific substances or simple mixtures having distinctive solubilities were separated and much new information was gained about the kinds of chemical substances contained in plants His system was modified by later chemists and by the year 1800 Thomas Thomson Regius Professor of Chemistry at Glasgow was able to list twenty-one organic substances which had been derived from various plants and from animal tissues From Rouelle s time to the present chemists have followed his principle in seeking to discover unknown constituents of the materials in organic nature It was the beginning of food analysis

2 Papin s Pressure Pot and its Influence on Chemical Thought

In 1679 Denis Papin[8] invented a pot with a close fitting lid securely fastened and equipped with a safety valve Its original purpose was the preparation of gelatin from bones Hitherto no one had heated organic substances with superheated steam The instrument was of great interest to chemists and soon various kinds of animal tissues—bone muscle nerve cartilage ligament horn etc —were separately heated with superheated steam When the brown liquor formed by this process was evaporated it appeared to the uncritical eyes of chemists of that period that every animal tissue yielded gelatin The idea was accepted that animal tissues of different appearance and properties were derived from gelatin combined with different proportions of water Albrecht von Haller the great Swiss physiologist (1707-1777) expressed his belief that about half the human body was gelatin Upon the evidence presented this conclusion seemed warranted

3 Beccari s Observations on Wheat Gluten

In 1742 Beccari[9] prepared wheat gluten by washing the starch out of dough Since it easily putrefied like flesh he concluded that it was animalized matter and drew the further deduction from the evidence that man with the exception of the spiritual part of his being was made from the animalized matter of his principal food wheat Sixty four years later Vauquelin and Fourcroy leaders in chemical thought and experiment after discovering that beans were rich in animal matter attributed to this vegetable product high nutritive value because of its high content of *aliment* from which blood could be formed and which could therefore nourish other tissues Their criteria for identifying "animal matter were the odour on burning precipitation by nut galls and certain heavy metals and susceptibility to putrefaction.

Common observations of animals losing their accumulation of fat when underfed or starved and its restoration when good feed was supplied early suggested that an animal could prolong life by expending its fat Lavoisier established by experiment

that respiration involves oxidation Starch had been known since Pliny's time (A D 23–79) It had been washed out of cereal grains for use as an adhesive Oils were pressed out of various seeds from early times The fact that ordinary foods contained starch and fat as well as animal matter was known to Lavoisier

In 1811 Gay Lussac and Thenard devised the first method for quantitatively determining the percentages of carbon hydrogen and nitrogen in organic compounds The method was not accurate but was improved by several chemists during the next thirty years A new era was thus opened and increasing numbers of organic substances occurring in foods were subjected to chemical analysis for their content of these elements Especially important was the observation of the high nitrogen content of animal tissues with the exception of fat In 1841 Liebig[10] the most influential chemist of his generation suggested that the nutritive values of foods could be estimated on the basis of their nitrogen content The albuminous or flesh forming food he called plastic food In his view the essentials of an adequate diet were sufficient *plastic* (protein) and fuel foods (carbohydrates and fats) the former to build muscle and other body tissues and to furnish energy for muscular work the latter through respiration (combustion) to keep the body temperature normal under conditions of cooling

About the year 1840 Mulder[11] interpreted his experimental observations to signify that there was but one *protein* in nature and that such contrasting albuminous substances as casein of milk fibrin of blood and egg albumen differed from one another because in them protein was combined with different amounts of phosphorus or sulphur or both to form protein compounds Liebig accepted this view It was also accepted by the earliest and later agricultural chemists who studied animal nutrition and was abandoned by notable investigators only toward the end of the nineteenth century

IV THE CHEMICAL ANALYSIS OF FOODS

In 1860 Henneberg and Stohmann (1885)[12] director and chemist, respectively of the first agricultural experiment station to be supported at public expense (Weende near Gottingen) adopted the most important ideas which earlier chemists had applied to the analysis of plant substances They devised a system of food analysis which became standard practice throughout the world for half a century By this method a sample was dried to determine its moisture content Another sample was extracted with ether to remove what was called crude fat Another was used to determine its nitrogen content and another was burned to estimate its ash The nitrogen found was multiplied by a factor 6 25 and the result was called protein A sample was digested successively with dilute acid and dilute alkali and the part which was not dissolved was called crude fibre This consisted principally of cellulose and lignin which were supposed to be unchanged in the digestive tracts of animals The sum of the protein fat ash and crude fibre was subtracted from 100 to obtain a fraction called nitrogen free extract which was thought to represent utilizable carbohydrates For half a century chemists and physiologists assumed that this analysis represented like nutritive worth in foods regardless of their source e g seeds roots tuber or leaf of different kinds of plants and that protein and fat from milk egg and meat had the same nutritive values as those from vegetable substances Tens of thousands of analyses were made of foods and feeds grown on different soils in different geographical areas The data were believed to be important for planning rations for farm animals and for assessing the values of human dietaries

Meanwhile agronomists were occupied through five decades with studying the cost of producing protein and energy in different farm crops The hope was entertained that when sufficient data were available farmers could use tables of food composition for calculating animal rations so as to secure the nutrients at the lowest cost and so realize the greatest profit in animal production The results gained from this system of judging food values were disappointing and by 1890 the chemical analysis had fallen into disrepute However not even the most experienced chemists and physiologists ventured suggestions for remedying the difficulty

At the end of the nineteenth century protein and energy requirements of human subjects for various kinds of work and of domestic animals for growth and for milk egg and wool production dominated the discussion of nutrition in agricultural bulletins and in books on diet in relation to health For another decade a vigorous controversy was carried on over the allowance of protein which best promoted health (McCollum 1957b)[13] Voit Atwater Benedict and Sir James Crichton Browne believed in the merits of high protein consumption in the interest of physiological well being while Chittenden Irving Fisher and J H Kellogg in America and Hindhede in Denmark, urged abstemiousness in protein-eating The views of the former group received strong support from a study by McKay *The Protein Element in Nutrition* (1912) He assessed a number of racial group dietaries of

people in India on the basis of their protein content only and attributed the high standards of physical vigour of some groups to high protein consumption and the poor physical status of other groups to deficiency of this element in their diets

Throughout the nineteenth century little attention was given to the physiological importance of the in organic moiety of dietaries A few experimental tests were made by animal husbandmen to find to what extent the bones of domestic animals could be in creased in size and breaking strength by the use of supplements of bone meal calcium carbonate or wood ashes Bone size and strength were improved by this means Acid base balance was considered by von Bunge to be important in nutrition Still earlier the need of animals for sodium chloride had been discussed for opinions differed on the subject That an ash free diet could not support life of animals was first proved by Forster in 1873 In 1889 in his famous text book of physiological chemistry G von Bunge discussed potassium only in relation to the need for sodium chloride by grazing animals whose intake of potassium was high because of their ingestion of potassium rich plants Sodium salt he believed was essential for assisting herbivores to excrete the excessive intake of potassium Plant physiologists in the nineteenth century were far in advance of animal physiologists in seeking to determine inorganic requirements It was not until after 1920 that it emerged that this was a field of great importance

V THE DISCOVERY OF VITAMINS

The first man to suggest that a diet which supplied only protein carbohydrate and fat was inadequate for the support of life was J B A Dumas[14] the famous French chemist In 1871 he described in the siege of Paris the accompanying shortage of food especially for infants and young children The high mortality among these was attributed to the shortage of milk and eggs Efforts were made to construct artificial milk by making an emulsion of fat in a solution of albuminous substance and sugar When given this milk substitute infants soon died No one knew why Dumas declared that no chemist could justly claim to be able to make a food equivalent to milk by such means He clearly expressed his belief that there were in milk chemical substances still unknown and of nutritional significance He did not follow up this observation by experimental inquiry into the nature of these hypothetical constituents which were so impor tant to life

First to restrict animals (mice) to a diet consisting of purified protein (casein) milk sugar and milk fat together with what were believed to be the essential inorganic elements was N Lunin (1885)[15] At the suggestion of his famous teacher von Bunge he tried feeding this simplified diet of purified food substances to determine whether the animals would die of acid intoxication from sulphuric acid and phosphoric acid formed from oxidation of the phosphorus and sulphur in the casein when alkali bases were not present in sufficient amounts to neutralize them On this diet the mice soon sickened and died whereas when he gave other mice only milk as food they remained in appar ently normal condition for sixty days Lunin stated that milk must con ain essential nutrients other than the principal constituents casein lactose and milk fat and that they were in the whey constituents Everything that Lunin observed and concluded from his results can be deduced from the observations of Dumas on the fate of infants fed synthetic milk "

Space does not permit giving an account of twelve experimental inquiries published between 1881 and 1906 on simplified diets the chemical constituents of which were known The author has given a description of these elsewhere (McCollum 1957a)[1] Each investi gator met with failure to nourish mice or chickens on such diets In 1844 Gobley discovered lecithin in egg yolk and found that it contained both nitrogen and phosphorus In 1881 Miescher isolated nucleoprotein from the heads of salmon sperm Hoppe-Seyler had prepared a similar substance from pus from an abscess and in 1874 Picard observed that the purines guanine and xanthine were liberated on hydrolysis of nucleo-proteins A few men who were curious about the cause of failure of mice on diets made of purified food substances naturally wondered what was missing and so efforts were made to supplement such mixtures with lecithin or with nucleic acids These additions did not improve the status of experimental animals

The step taken by Pekelharing in 1905[16] to demon strate the extreme importance of small amounts of unidentified nutrients was the most meaningful to that date He observed that while mice could not survive many days on a diet of purified food substances they were greatly improved in health by a small allowance of whey This contained so little of anything which was recognized as a food principle that Pekelharing con cluded that unidentified nutrients existed in the whey He did not influence the thought of any investigators of his generation

After Pekelharing the most important experiments with foods were carried out by Rohmann and reported in 1908[17] He proved that young animals failing in health on a diet of protein carbohydrate fat and salts were greatly benefited by small supplements of several

kinds of natural foodstuffs This clearly pointed to the existence in natural foods of unidentified nutrients but his results had no immediate influence on the course of nutrition studies He did not attempt to inquire into the nature of these substances

In 1907 McCollum[18] undertook to test the possibility of turning failure into success by making diets of purified food substances more palatable Failure again resulted However by good fortune he prepared an experimental diet the milk sugar of which contained unrecognized impurities derived from whey His experimental rats also had access to their faeces which they ate in considerable amount and from which they derived unidentified substances On this basal diet young rats soon sickened and died when the fat supplied was lard or olive oil but were able to grow for a time when butter fat or egg yolk fat was used as a supplement This was the earliest conclusive proof that certain fats contain an essential nutrient which is absent in others They were able to transfer this new something from butter fat to olive oil by making soap from butter oil and shaking olive oil with this soap solution to form a fine emulsion The emulsion was broken with ether and the olive oil recovered in this solvent Feeding tests showed that it had acquired the nutrient property of the butter fat This factor was called vitamin A

Mention of the great discovery of Eijkman and Grijns[19] in 1896–1901 is made at this point because although in the perspective of history it was epoch making it did not influence thought by nutrition investigators until a decade later Eijkman observed that chickens fed solely on polished rice developed multiple neuritis with head retraction and paralysis and that when given unhusked rice they quickly recovered Water or alcohol extracts of rice polishings when administered to helpless birds cured them dramatically He noted the similarity of symptoms of birds with the rice sickness to those of human subjects with beriberi then a disease causing high incidence of disability and mortality among peoples whose staple article of diet was polished rice As the explanation for the aetiology of the disease he postulated the existence in the endosperm of rice kernels of a nerve poison for which there was a pharmacological antidote in the outer layers of the grain Vordermann[20] eradicated beriberi from prisons and asylums where it was very common by changing the diet of the inmates from polished to unpolished rice Grijns (1901)[19] correctly interpreted the phenomena as due to a deficiency of some essential nutrient There were however a number of dissenting opinions concerning the cause of beriberi and it was not until the publication in 1910 of the studies of Fraser and Stanton[21] that all were forced to accept the deficiency hypothesis

These studies were all published in journals devoted to hygiene or medicine not usually read by chemists The present writer was unaware of them until the appearance of E B Vedder s Cartwright Prize Essay *Beriberi* in 1913[22] and Funk s volume *Die Vitamine*[23] in 1914 The latter received wide publicity because in it Funk proposed his vitamin hypothesis This postulated the existence of a number of unidentified nutrients a deficiency of one causing beriberi another rickets another scurvy another pellagra etc

In the first decade after 1900 text books by Lusk and Sherman and bulletins on foods and nutrition published by the U S Department of Agriculture in which Atwater was the leading authority still taught that protein carbohydrate and fat were the only nutrients which need be emphasized in assessment of quality in diets The view taken of mineral elements in nutrition was that any mixed diet which provided sufficient of the organic dietary essentials would doubtless contain enough inorganic substances

VI. NEW VIEWPOINTS ON PROTEIN NUTRITION AFTER 1900

About the year 1900 the protein element in nutrition assumed new aspects Had someone written a good history of the progress of thought and experiment in animal nutrition and the chemistry of foods progress would have been advanced by more than a decade It has been mentioned that Mulder Liebig Voit Henneberg and their followers assumed that protein from every source had the same nutritive value That this could not be true had been shown in 1818 by Braconnot[24] who reported that one kind of amino acid glycine was crystallizable from the mixture formed by hydrolysis of gelatin and another kind leucine crystallized from the mixture derived from hydrolysis of muscle or wool with sulphuric acid Further evidence that proteins differed in composition was reported in 1872 by O Nasse[25] who hydrolysed with sulphuric acid several kinds of proteins from plant and animal sources then made the solution alkaline and distilled and measured the ammonia which came over The amounts of ammonia yielded by his samples differed so much that there could be no doubt that proteins manifested strongly contrasting chemical composition This being the case reflection should have convinced investigators that they had different nutritional values These facts were completely ignored for many years

In 1886 Schulze and Steiger[26] first used phosphotungstic acid to precipitate an amino acid from an aqueous extract of germinating seeds From the precipitate they isolated arginine Drechsel Kossel and

Hedin later employed this reagent for precipitating basic amino acids and discovered lysine and histidine *Hausmann (1900)*[17] *applied the method of Nasse* and of Schulze and Steiger to measure the amounts of ammonia and of basic amino acids in several protein hydrolysates The results were so contrasting with respect to both these types of nitrogenous substances that it convinced biochemists that proteins must have different nutritive values since certain of them were so differently constituted

Willcock and Hopkins[18] in 1906 demonstrated by experiments with mice the importance of the amino acid tryptophan in nutrition Hopkins and Cole had recently discovered it and had found that it gave a certain colour reaction with a reagent which had long been employed as a test for protein Zein the principal protein of maize, did not give this colour test which indicated that it did not yield tryptophan on digestion They employed a synthetic diet in which the sole protein was zein Mice were able to survive on an average only fourteen days on the basal diet but with a supplement of tryptophan they survived twenty eight days

About 1902 E Fischer devised a method for fractionally distilling the ethyl esters of various amino acids contained in protein hydrolysates Partial separation of amino acids was effected by this means Although it was far from giving quantitative results this method of analysis clearly showed that different *proteins yielded strongly contrasting amounts of* various amino acids This confirmed the conclusions of others mentioned above In the light of the new observations it became evident that the nutritive values of proteins depended on their yield of amino acids in terms of the nutritive needs of the body for each Evidently nitrogen multiplied by a factor did not repre *sent the same nutritional values in proteins from* different sources

In 1909 Osborne and Mendel[19] began their experi ments on Osborne s collection of purified proteins Using rats as their subjects and growth and reproduc tion as criteria they sought to determine the relative values of a wide variety of proteins They first at tempted to use a *diet of purified food substances* Having failed in this they next used a diet containing 28 3 per cent of whey solids prepared by heating acidified whey to coagulate lactalbumen filtering and evaporating the filtrate to dryness This adjuvant provided all water soluble vitamins and a favourable mixture of inorganic compounds together with some vitamin A By feeding young rats this basal diet to which different purified proteins were added they dramatized the fact that proteins were indeed, as different in biological values as the chemical analyses had suggested they should be The studies of Hopkins and of Osborne and Mendel formed one of the monuments on the path of nutrition investigations.

I THE BIOLOGICAL ANALYSIS OF CEREALS

In farm practice in America pigs were often fed exclusively on corn (maize) on which they did not make satisfactory gains In 1912 McCollum *(1957d)*[19] undertook to determine whether it was possible to identify the nature of the shortcomings of this cereal when it served as the sole food for rats Amino acid requirements and different composition of proteins were then much discussed The discovery of the fat soluble vitamin A afforded another factor the distri bution of which it was desirable to study McCollum had noted that the ashes of all cereals were very low in calcium and might not be adequate for growing pigs *Animal husbandry studies had shown* that bone size could be improved by supplements of calcium compounds

On the basis of these ideas McCollum compared the effects on young rats of feeding (1) corn alone (2) corn plus a calcium salt (3) corn plus a protein (casein) to provide additional amino acids (4) corn *plus butter fat to provide the new fat soluble nutrient* Rats given rations (2) (3) and (4) were distinctly better nourished than on ration (1) When they were given corn plus both calcium and protein or calcium and butter fat or protein and butter fat they were in decidedly better condition than when given only one supplement but they still did not grow at the maxi *mum rate nor present the healthy appearance* which they presented when given all three supplements These results showed that several dietary factors in corn were present at far below optimum levels. Comparable experiments with wheat and rolled oats demonstrated that the three cereals were similar in their dietary properties They were all improved by *the same supplements*

The response of young rats to rolled oats plus the three supplements was less satisfactory than with wheat and corn The cause of this remained unexplained for some years It was eventually shown to be due to the riboflavin deficiency of the oat kernel.

VII SUPPLEMENTARY RELATIONS AMONG COMMON FOODSTUFFS

McCollum and his co-workers investigated the effec tiveness of various combinations of natural foods They observed that combinations of two or more cereal grains when fed to young rats were not superior to the grains fed singly This was easily explained on the basis of their having like deficiencies. But the combination of a cereal grain with the leaf of a plant (alfalfa leaf flour) yielded a mixture of nutrients which

nourished young rats far better than did any combination of seeds It became clear that the leaf of a plant is very differently constituted as a source of dietary essentials from any seed and that it serves as an excellent supplement to the latter This discovery afforded an explanation of the soundness of the practice of some farmers of feeding growing pigs corn or other cereal while allowing them daily access to green forage plants as pasture The experiments with rats demonstrated no new fact but afforded an understanding of the basis of a sound agricultural system of swine production already believed in by some intelligent farmers These new viewpoints McCollum presented in a lecture before the Harvey Society (New York) in 1917[31]

1 Dietary Deficiencies of Polished and of Whole Rice

In 1915 McCollum and Davis[32] reported the results of supplementing polished rice with the same additions that improved whole rice and other cereals as food for the growing rat They found that these supplements were insufficient for polished rice In addition a water extract of some natural food was necessary to support well being in the animals Since they had become acquainted in 1913 with the studies of Eijkman Grijns *et al* through the publication of E B Vedder s Cartwright Prize Essay *Beriberi*[32] and Funk s *Die Vitamine*[3] they assumed that the water soluble substance required by rats fed polished rice supplemented with chemically characterized nutrients was identical with the anti beriberi factor of the Dutch investigators After further experimenting McCollum and Kennedy (1917) asserted their belief that only two unidentified food factors existed viz the fat soluble factor vitamin A and the factor preventive of beriberi

The two-vitamin hypothesis was widely accepted for a few years However H H Mitchell A G Hogan and A D Emmett working independently produced convincing evidence that at least two unidentified dietary factors were contained in the water extracts of natural foods The time had arrived when interest in nutrition studies was to increase greatly and at a rapid rate and the new viewpoints were voiced by several men who collectively broadened the outlook in this field These will be briefly mentioned

2 A F Hess s Contribution to Knowledge of Infantile Scurvy

From the observations of Lind (1753) it had been known that scurvy in man could be prevented or cured by fresh fruits or vegetables and Holst and Froelich had produced experimental scurvy in guinea pigs in 1907 by dry or cooked diets but the medical profession even as late as 1915 was not in general taking measures to prevent scurvy in infants since it had but recently appeared After about 1890 ordinances requiring pasteurization of milk sold in cities became common and bottle fed babies often developed the disease About 1914 A F Hess[33] paediatrician to a foundling home saw many cases of scurvy among his patients who were fed pasteurized or boiled milk and barley water Influenced by the work of Holst and Froelich he rediscovered the fact that heated foods for infants must be supplemented with fresh fruit or vegetable juices in order to safeguard the health of the blood vessels Having convinced himself of the widespread error in the feeding of bottle babies and young children then common he devoted himself to making known his system for safeguarding infants against scurvy The fact that rats swine and other animals generally employed for experiments with foods at that period remained in health on dried foods confused most investigators of nutrition

3 Pellagra Proved to be a State of Malnutrition

Although pellagra had been long known it did not constitute a great human scourge in the United States until after 1900 Opinions among medical men differed widely as to its cause The theories that it was caused by eating mouldy maize that it was an infection and that it was a manifestation of sensitization to sunlight were the principal ones discussed

First to describe the cure of pellagrins by dietetic treatment was Voegtlin (1914)[33] But the conclusion of the Thompson-McFadden Commission after several years of epidemiological study that the disease was caused by the bite of the stable fly prevented Voegtlin from making much impression on medical opinion Joseph Goldberger was the outstanding investigator who supplied incontrovertible evidence that the disease was caused by some deficiency in the diet (1915*a* *b* 1916 1925 1928*a* *b*)[34] The experimental study of pellagra in man was complicated by the frequent occurrence of riboflavin deficiency among sufferers from the disease and also by the development of bronzing of the skin only when it was exposed to sunlight Hence symptoms of pellagra were not uniform and accordingly clinicians were confused in their diagnoses

As in the case of other deficiency diseases later to come to light the solution of the pellagra problem was achieved through the experimental production of the disease in an animal followed by systematic study of the factors involved in its aetiology First to discover in dogs what he believed to be the analogue of pellagra in man was T N Spencer a veterinarian in North Carolina In 1916[35] he reported his conclusions concerning the cause and nature of blacktongue in dogs and described the cure of the

disorder by giving the animals milk meat and eggs Since he published his study in a veterinary journal it failed to attract attention That blacktongue in dogs was analogous to pellagra in man was rediscovered by Goldberger[36] twelve years later He showed that it could be prevented or cured by certain diets It was not until 1937 that Elvehjem and his associates[37] demonstrated that blacktongue in dogs was dramatically cured by administration of nicotinic acid In 1938 Spies and his associates Street and Cowgill Margolis Smith Dann and others proved that nicotinic acid (niacin) or its amide was a specific remedy for pellagra in man Nicotinic acid had been isolated from an extract of rice bran by Suzuki in 1912[38] It was isolated by Subbarow in 1937 from liver

For some years there was confusion about the nature of what Goldberger considered to be rat pellagra The disorder was not cured by niacin It was later renamed by Gyorgy rat acrodynia and was found to be due to deficiency of pyridoxin In 1938 Chick and her associates[39] observed that young rats thrived on certain experimental diets which induced pellagra in man swine and dog They devised a diet deficient in both niacin and the amino acid tryptophan and found that when restricted to it rats developed a disorder which could be cured by *either* niacin *or* tryptophan This was pellagra in the rat Chick showed by her experiments that tryptophan can be converted into niacin in the body

4 Further Progress in the Discovery of Fat Soluble Vitamins

In 1919 E Mellanby[40] demonstrated that the diet could be so adjusted as to be the factor determining whether puppies developed rickets or not He offered experimental evidence that some fat soluble unidentified factor could prevent or cure the bone disorder He tentatively suggested that deficiency of vitamin A might be the cause of rickets Hitherto it was widely believed that bad hygiene climatic conditions and lack of exercise caused the disease Mellanby listed twelve foods which did not protect and eleven foods which did protect puppies against rickets Among the latter were olive oil lard meat extract and malt extract which did not contain vitamin A so it appeared uncertain whether the anti rachitic factor was vitamin A

In an experimental study made between 1918 and 1922 by biochemists and paediatricians at Johns Hopkins University[41] it was demonstrated that an important factor in causing young rats to develop severe acute rickets was the ratio between calcium and phosphorus in their food If one of these elements was in low concentration and the other disproportionately high (*e.g* 0 2 and 1 2 per cent respectively) bone growth was abnormal as shown by histological studies provided that (1) the animals were deprived of certain fats and (2) they were kept out of direct sunlight (in shadow or behind window glass) Opportunity for exercise was unrestricted Pursuing the analysis of the properties of the different food substances in their experimental diets McCollum, Simmonds Park and Shipley concluded (1922, 1923)[41] that a second fat soluble vitamin existed Its physiological function was especially marked in preventing rickets in young rats kept on rickets producing diets by regulating the content of inorganic phosphate ions in the blood plasma Since at that time accessory food factors (vitamins) A B (anti beriberi factor) and C (anti scorbutic factor) were known the anti rachitic factor was called vitamin D It was found to be abundant in cod liver oil much less so in butter fat, and absent from many vegetable oils and the adipose deposits of mammals Animals in direct sunlight while kept on rickets producing diets had no bone defects.

It was later shown by Hess (1924)[42] and by Steenbock and Black (1924)[43] simultaneously that when rachitogenic diets were exposed to ultraviolet rays, vitamin D was generated Its precursor was shown to be present in certain fats and was soon traced to the non saponifiable fraction and then to the sterols contained in it

At this stage studies by several groups of biochemists were directed to identifying the substance in the sterol fraction from fats which was susceptible to conversion into vitamin D by ultraviolet rays Space does not permit of giving the details of these In 1927 Rosenheim and Webster[44] in England and Windaus (Germany) and Hess (America)[45] simultaneously reported that the precursor of vitamin D is ergosterol This is the principal sterol in oil of ergot yeast and other fungi but is widely distributed in nature in small amounts Certain other sterols when activated were found to possess anti rachitic properties hence several forms of vitamin D are known

McCollum and his associates devised the so-called *line test* for accurate assay of the anti rachitic potency of oils etc (1922) Using this test Bills assayed many kinds of fish liver oils and found that there is enormous variation in their vitamin D potency Cod liver oil of Pharmacopoeial grade contains about 100 International Units of vitamin D per gramme Blue fin tuna liver oil contains about 40 000 I U and yellow fin tuna liver oil about 10 000 I U per gramme

Cod liver oil had been employed as a remedy for the cure of rickets since the report of Scheutte (1824) that it was effective for this purpose But medical opinion differed as to its value and the controversy over the aetiology of the disease tended to prevent its wide spread use. With the discovery that rickets is a disease due to lack of a vitamin and the identification of

certain sources from which it might be had paediatricians everywhere began to prescribe antirachitic therapy for all infants and young children with the result that rickets has become a rare disease

5 H M Evans and Associates Discover Vitamin E

Sterility premature delivery and foetal death before or at term had been repeatedly observed by nutrition investigators but it was not until Evans s experimental studies that one cause was definitely shown to be deficiency of a hitherto unsuspected nutrient substance X later named vitamin E (1922)[46] On certain diets which supported normal growth to maturity and ovulation in rats and on which they were able to conceive gestation invariably failed Rats kept on such diets and mated to normal males became pregnant but the foetuses showed signs of retarded development on the eighth day of gestation By the twelfth day there appeared defects in the vascular system in the foetal liver deficiency of blood islands in the yolk sac and of erythroblasts in the heart and blood vessels Death of the foetuses occurred on the thirteenth day as the result of asphyxiation Administration of a concentrate of vitamin E as late as the fifth day after mating caused dramatic salvaging of the foetuses and enabled the mother to deliver normal young at term The great merit of Evans s studies was his introduction of histological techniques into the study of the nature of the defects which were caused by deficiency of vitamin E These were the earliest studies of their kind

In 1927 Evans and Burr[47] traced a type of paralysis in young rats to deficiency of vitamin E Goettsch and Pappenheimer[48] described muscular dystrophy in rabbits and guinea pigs kept on certain experimental diets and found that administration of vitamin E preparations had curative properties Later they concluded that this observation was in error and expressed the belief that the disorder must be assigned to some other cause Einarson and Ringsted[49] described lesions in the nervous system in E-deficient animals Pappenheimer and Goettsch (1931 1934)[50] described the development in birds of encephalomalacia when they were restricted to the same type of diet that had caused muscular dystrophy in rabbits and guinea pigs Mason observed the abnormalities in development of germ cells in E-deficient rats and Barrie (1937 1938)[51] and Verzar (1931)[52] described histological changes in bones thyroid and the anterior lobe of the pituitary These studies together with Evans s account of blood vessel defects in foetuses revealed that in deficiency of this nutrient multiple abnormalities develop In 1940 C G Mackenzie and associates[53] carried out extensive experiments with rabbits restricted to diets in which the only deficiency was vitamin E, which had already been identified by Evans and his associates (1936)[54] as α tocopherol Mackenzie and co-workers demonstrated beyond question that muscular dystrophy in this species results from uncomplicated deficiency of this factor

Thirty years of experimental studies of the histopathological lesions of vitamin E deficiency have shown that these are numerous There are morphological changes in several unrelated tissues and they seem not to be associated with dysfunction of any specific type of cell or tissue There was a long debate on whether or not vitamin E plays any role in human metabolism The evidence presently available warrants the conclusion that should a human subject at any age become sufficiently depleted of this nutrient tissue alterations and perversion of function unmistakably due to its absence would result

Of outstanding interest is the discovery by Gyorgy and his associates (1949 1952)[55] that erythrocytes of adult rats deficient in E and newborn rats from mothers on normal diets are easily haemolysed *in vivo* and *in vitro* by low concentrations of dialuric acid alloxan or hydrogen peroxide and that small amounts of α tocopherol protect the cells against these reagents They found that the red blood cells of full term infants at birth show mild haemolysis when treated with minute amounts of hydrogen peroxide and that incubation of washed erythrocytes with α tocopherol makes them resistant to this effect Tocopherol fed to the infant accelerates the disappearance of the fragility of the cells which they observed to occur during the first week or so of post natal life They point out the practical clinical significance of this fact Gyorgy s observations have been confirmed by Gordon and his associates (1951 1952)[56] Excellent reviews of the scientific literature on vitamin E have been written by Mackenzie (1953)[57] and by Mason (1953)[58]

6 The Discovery of Riboflavin

It has been mentioned that nicotinic acid was a familiar substance to organic chemists for many years before its physiological significance was discovered Riboflavin is another with a comparable history It first attracted the attention of a chemist in 1879 (Blythe) and was described in some detail by Bleyer and Kallmann in 1925[59] Both investigators secured it from whey In 1933 Ellinger and Koschara[60] isolated from whey three dyes of red or reddish yellow colour which were rich in nitrogen and oxygen and showed strong yellow green fluorescence in aqueous solution They suggested that these three dyes were related to two oxidation ferments obtained from yeast by Warburg and Christian (1932)[61] and to others contained in urine Kuhn and his collaborators (1933)[62] prepared from egg white (180 mg from 10 000 eggs) a

yellow pigment having strong green fluorescence The name *flavins* was applied to this new group

The Warburg–Christian yellow enzyme was found to oxidize Robison s ester (glycopyranose-6-monophosphate) The enzyme was in this process reduced to the leuco form and could be regenerated by shaking the solution with molecular oxygen This series of observations revealed that it serves as an oxygen transport system between molecular oxygen and the substrate

In 1931 Bourquin and Sherman[43] devised a diet suitable for assaying for what was then known as vitamin B_2 or vitamin G these terms being used synonymously to designate an unidentified nutrient eventually called riboflavin It consisted of a mixture of casein salts butter fat cod liver oil starch and an 80 per cent alcohol extract of whole wheat in the proportion of extract from 50 grammes of wheat to 100 grammes of the food mixture When fed the basal Bourquin diet young rats from mothers kept on an experimental diet steadily declined in weight and health When given the same diet plus a supplement of riboflavin they grew approximately in proportion to the fraction of the minimum requirement of this vitamin they received This method constituted a fairly accurate assay procedure for riboflavin in foods

The symptoms of riboflavin deficiency in rats are dermatitis and loss of hair from head, abdomen around the nose mouth and eyelids and sealing of the eyelids by a sticky exudate The front paws are stained with blood from rubbing the inflamed margins of the nostrils and the abdomen is wet with blood stained urine

Symptoms of Ariboflavinosis in Humans

Spies *et al* (1940)[44] and Sebrell and Butler (1939)[45] and their associates described the symptoms of riboflavin deficiency in man These are cheilosis eversion of the lips fissures at the mouth angles scaly greasy desquamation about the nose and on the ears There is a sensation of roughness in the eyes with itching and burning and mild photophobia There is also invasion of the cornea by capillary blood vessels These symptoms have often complicated those of pellagra Only after riboflavin was discovered and its metabolic functions recognized was it realized that many patients with pellagra were in multiple deficiency states Riboflavin participates in no less than eighteen enzyme systems Small wonder that a deficiency of it results in profound metabolic disturbances

7 Discovery of Vitamin K

In the years 1929–31 Dam Horvath and McFarlane working independently observed delayed clotting time of the blood of chickens on certain rations Dam, in Copenhagen also noted extensive internal haemorrhages in chickens fed a certain experimental diet (1934)[77] He found that the blood of these birds was deficient in prothrombin The preventive factor he traced to the non sterol fraction of the non saponifiable fraction of pig liver fat To this unsuspected nutrient which he showed by experiments to be distinct from other fat soluble vitamins A D and E, Dam gave the name vitamin K

Dam s studies aroused great interest and several groups of biochemists undertook to isolate and identify the new dietary factor The details of experimental work planned with this objective cannot be described here In 1939 four groups of scientists working independently under the leadership of Almquist and Doisy (MacQuorquodale *et al* 1939)[78] (Almquist and Klose 1939)[79] (Fieser and Karrar 1939)[80] respectively identified several *naphthoquinone* derivatives which in different degrees possessed the properties of naturally occurring vitamin K

It was observed that guinea pigs suffered no reduction of prothrombin when fed the rations which produced this deficiency in chickens It was also found that bacteria growing in fish meal increased the vitamin K content and that chicken faeces allowed to stand some hours showed a marked increase of this substance Thus the bacterial synthesis of vitamin K was established The ability of micro-organisms in the alimentary tract of guinea pigs to synthesize the vitamin rendered these animals resistant to the effects of a K-deficient diet

Vitamin K proved to have great therapeutic value Delayed clotting time in patients suffering from obstructive jaundice was already known to surgeons and had been correlated with deficiency of prothrombin in the blood Administration of vitamin K pre-operatively promptly raised the prothrombin level of the blood and restored its clotting time to normal thus reducing the risk of surgical operations for relief of this condition It was also found to be a useful remedy for haemorrhagic retinitis and for preventing the haemorrhagic tendency in the newborn

VIII THE DISCOVERY OF THE ESSENTIAL FATTY ACIDS

Between 1840 and 1850 there was a controversy between certain French and German chemists over whether animals could synthesize body fat from carbohydrate taken as food Experiments sustained the view of Liebig that such synthesis was possible Proof rested on analytical data which showed that animals laid on more fat reserves in adipose tissue (pigs geese) or gave out more fat in milk (cows) than was contained

in their food Rubner concluded from his studies on energy metabolism that carbohydrate and fat were interchangeable in isocaloric quantities in animal metabolism His views were generally accepted for many years The fact that animals can synthesize fats and can use them as sources of energy as they do carbohydrates led nutrition investigators to believe that there was no physiological need for fats or fatty acids in the diet

The matter was not permitted to rest without attempts to prove by experiments the possibility of animals subsisting on fat free diets In 1913 the discovery that certain fats contained an unidentified essential nutrient led to speculation whether a fat free diet could support an animal in health Osborne and Mendel[44] attempted in 1920 to make a crucial test of the problem Their diet for experimental rats was not sufficiently depleted of fatty substances to afford conclusive proof They could only show that if young rats require a dietary source of any fatty acid it must be only in extremely small amounts

In 1929 Burr and Burr[47] devised an experimental diet which was essentially free from fats and on which young rats failed nutritionally A supplement of certain fatty acids caused their recovery They compared the values of fats from different sources and found that only those containing linoleic acid which contains two double bonds supplied the nutritive needs of animals Later studies by others showed that linolenic (three double bonds) and arachidonic (four double bonds) could replace linoleic acid in animal nutrition Indeed Turpeinen (1938)[48] proved that arachidonic acid is the indispensable nutrient and that it can be synthesized in the body when either of the others named is provided in the food The discovery of the essential nature of certain fatty acids was one of the classics of nutritional investigations

The symptoms of essential fatty acid deficiency in the rat are scaliness and eventual necrosis of the tail disturbances in the reproductive processes and kidney lesions

IX THE EMERGENCE OF INTEREST IN THE PHYSIOLOGICAL SIGNIFICANCE OF INORGANIC ELEMENTS IN NUTRITION

1 Iodine

The element iodine was discovered by Courtois in 1811 and in 1820 Coindet used it in treatment of endemic goitre then common in parts of Switzerland He suspected that the therapeutic virtues which had long been attributed to the ash of sponge as a remedy for goitre were due to this element Coindet and also Boussingault from observations on Indians in South America asserted their belief that iodine administration could prevent or cure goitre But excessive dosage did harm to patients and brought this remarkable specific against the disease into disrepute

Interest was renewed by the discovery by Baumann in 1896 that the thyroid gland contains much greater amounts of iodine than any other tissue The studies of Marine and others after 1908 established the fact that iodine is an essential nutrient and that in great areas in several parts of the world the amount of iodine in water and soils is too small to supply the needs of the human and animal populations This deficiency results in perversion of thyroid function The provision of a supplement of iodine in the form of iodized salt was found to be a cheap and effective means of preventing goitre

2. Copper

Copper was observed as early as 1840 to occur in the ash of healthy bodies and in 1847 Harless[49] discovered that it occurred in the blood of molluscs In 1848 Bert identified it as a constituent of the respiratory pigment in several lower forms of life Botanists learned about 1917 that application of copper compounds to fruit trees in certain areas could prevent or cure the disease *exanthoma* But it was uncertain whether this was a deficiency disease of the trees or a poison from an infecting agent Anna L Sommer in 1931[50] first proved that copper is a necessary nutrient for plants

In 1925 Hart and his associates[51] restricted young animals to milk as their sole food and noted the development of anaemia Administration of iron compounds did not cure the anaemia but the ashes of corn lettuce and especially of liver cured or prevented deterioration of the blood when milk which is very poor in iron copper and manganese was the sole food They traced the effectiveness of the ashes named to their content of copper They proved that copper is an essential nutrient in the absence of which iron cannot be utilized for conversion into haemoglobin This discovery had great influence on the thinking of nutrition investigators and led to many experimental studies designed to reveal other inorganic elements which might possess unsuspected physiological significance These investigations brought to light that the trace elements described in the following pages must be supplied by the food

3 Manganese

Manganese deficiency in young rats causes testicular degeneration in males and the production of non-viable young In chickens and turkeys during the

growing period deficiency of manganese causes skeletal defects called slipped tendon or perosis and low egg hatchability

4 Zinc

Zinc was shown by Raulin[72] in 1869 to be essential for growth of the mould *Aspergillus niger* but the significance of his observation was long overlooked The pathological effects of zinc deficiency in plants were described by Reed and Dufrenoy[73] in 1935 In 1937 Hubbell and Mendel observed beneficial effects on young rats from giving them zinc Hart and his associates (Todd *et al* 1934)[74] demonstrated the indispensability of zinc in animal nutrition

Zinc is a constituent of carbonic anhydrase an enzyme which in the blood catalyses conversion of carbonic acid to carbon dioxide and water It is associated with insulin and five other enzymes Since each of the steps in metabolism which these enzymes catalyse is an essential molecular change it is obvious that deficiency of it causes profound injury Zinc is positively correlated with thiamin in foods and it may be that a deficiency of it as well as of thiamin is a factor in the polyneuritis syndrome Hart and his associates (Wachtel *et al* 1941)[75] found the uric acid content of the blood of zinc-deficient rats to be twice the normal value and it could be reduced to normal by administration of zinc Salmon and Tucker have described the occurrence of zinc deficiency in swine on a certain farm ration and its remission upon the provision of the element Severe dermatitis and diarrhoea were prominent symptoms Other than this instance deficiency of zinc has been seen only in laboratory animals

5 Cobalt

Cobalt deficiency results in a type of nutritional anaemia in sheep and cattle In Australia it is known as Morton Mains disease and as coast disease, and in other parts of the world as pines bush sickness, etc. A sheep may develop microcytic hypochromic anaemia and die for want of 1 mg of cobalt a day This fact was established by Underwood Filmer Lines and Marston in the years 1934 and 1935[76] They observed the deficiency in sheep and cattle which grazed on herbage grown on cobalt-deficient soils Several years of intensive investigations were required to reveal that cobalt was the missing nutrient Administration of this element sufficed to protect animals against anaemia, or to cure those which had developed the disease Hitherto no one had suspected that cobalt was of any biological importance Only ruminants are able to utilize inorganic salts of cobalt, man omnivores and carnivores must obtain it in the form of an organic compound which will be considered later

X. GROWING INTEREST IN NUTRITION INVESTIGATIONS

With each new discovery of a chemical substance which the diet must provide if an animal is to maintain health the advancing science of nutrition rose in the estimation of chemists physiologists pathologists and clinicians The number of investigators who devoted themselves to the study of specific problems in this field rapidly increased In 1931, 724 senior authors published 3 024 papers which were abstracted in *Nutrition Abstracts and Reviews* The most notable investigations were those directed toward discovering hitherto unsuspected nutrients both organic and inorganic

With the demonstration of the existence of each new vitamin (the description of symptoms in an experimental animal which developed because of its deficiency or lack in the diet and their disappearance upon its provision) investigators followed the pattern of formulating diets which contained every known nutrient and were fully characterized chemically and tested their effects on animals It was the common experience that failure to thrive and short period of survival resulted making it evident that still other essential constituents of the diet existed Testing for effective supplements for such synthetic diets revealed that for young rats and chicks water extracts of such foods as yeast wheat germ egg yolk liver, alfalfa leaf flour contained the substance or substances needed to make them adequate They were therefore water soluble and not fat soluble This fact directed the attention of the most constructive thinkers to the rewards to be reaped from testing the values of fractions of the water soluble material separated from natural foods for enhancing the value of experimental diets containing only known substances All nutrients soluble in water except the antiscorbutic vitamin C, were early designated the B-complex of vitamins Fractionation of this complex led to the discovery that it was unexpectedly complex.

Determination of the nature of the essential nutrients was a chemical problem Only biochemists could initiate animal experiments directed to this objective But since malnutrition of any type leads to alterations of physiological functions and pathological changes in anatomical structures these chemists were not competent to examine minutely so as to distinguish concurrent symptoms which together indicate the nature of the specific diseased states resulting from deficiency of individual dietary essen

tals Diseases of nutritional origin involve initially failure of a chemical interaction between two substances at the molecular level Pathologists and physiologists of the early twentieth century had little training in chemistry and could not visualize metabolic processes in terms of complex and varied concatenated chemical reactions Both the latter sciences were making rapid progress by the employment of other techniques than chemistry What was most needed after about the year 1900 was more co-operation between physiological chemists anatomists pathologists and physiologists in the study of the details of what happened to an animal in nutritional failure from different causes Such co-operation did not immediately appear rewarding but three investigators who were not chemists and who applied their special knowledge to the study of the pathology of malnutrition caused by specific dietary deficiencies made contributions which raised the standards of such work above what unaided chemists could achieve

Not much of value came from the early attempts to study the vitamin problem by observing animals restricted to polished rice as their sole food because in general unless the experiments were of short duration multiple deficiencies developed

In 1922 S Mori[81] an ophthalmologist working in McCollum s laboratory using the entire contents of the bony orbit described the histological changes in consecutive sections of specimens of the eye and its related structures in rats in different stages of depletion of vitamin A He described the progressive keratinization of epithelia and attributed all pathological changes in the eye to this change and to the secondary effects of drying of the conjunctival sac owing to destruction of the specialized epithelium of the tear gland

Evans an anatomist (1922)[82] applied histological techniques to the study of the placenta and foetal structures of guinea pigs and rats suffering from vitamin E deficiency He drew the attention of biochemists to the need of more searching techniques in nutrition studies Hitherto only the most obvious gross changes had been recorded in descriptions of the mode of failure of animals on inadequate diets

In 1926 Wolbach and Bessey[83] applied the special techniques and interpretation of pathologists to the study of the successive changes which occur in rats as they develop the abnormalities caused by deficiency of riboflavin These fine studies made clear the great opportunities which awaited investigators equipped to employ increasingly searching methods of investigation based on the techniques of chemistry and pathology to the study of malnutrition caused by diets deficient in different nutrients The application of the new and broader concepts to animal experimentation led to the discovery of the following additional members of the B-complex

1 Fractionation of the B complex Vitamins

In 1937 Gyorgy[84] distinguished three kinds of dermatoses in the rat which could be produced by faulty diets and cured by appropriate dietary supplements In 1939 Oleson and his associates[85] distinguished four such syndromes which were clearly referable to dietary errors One of these was traced to deficiency of a substance to which the name biotin was given This substance was for a time known as Bios II vitamin H and co-enzyme R It was discovered through a series of experiments directed toward finding the cause of a severe skin injury which afflicted rats fed raw egg white The condition was first described by Boas in 1927 Chief among the symptoms were eczematous dermatitis allopecia blepharitis spasticity and in some cases haemorrhages in the skin and oedema of the feet The injury from feeding raw egg white was soon confirmed by others in the rat and chick It was found that egg white contains a substance avidin which binds biotin and prevents its absorption from the digestive tract Deficiency of the vitamin then results

Biotin is essential for mammals and birds and also for the nutrition of yeast It was isolated by Kögl and Tonnis from boiled egg yolks and was synthesized by Harris and his associates[86] in 1943 The symptoms of biotin deficiency were produced experimentally in human subjects by Sydenstricker and his associates

(a) *Pyridoxin* was discovered as a result of studies of what was for a time called rat pellagra It did not respond to administration of nicotinic acid Gyorgy, who was prominent in determining its properties and its distribution renamed the disorder rat acrodynia Lepkovsky[87], Keresztesy and Stevens[88] and Gyorgy[89] working independently succeeded in crystallizing what had been variously known as Factor Y Factor I and vitamin B_6 It was named pyridoxin at the suggestion of Gyorgy

(b) *Pantothenic acid* was first recognized as the result of an attempt by R J Williams to use yeast as a test organism for thiamin (B_1) It was found that this was not the yeast growth substance studied by Wildiers who designated it Bios In his yeast studies Williams[90] eventually isolated and described pantothenic acid so named because of its wide distribution in nature It is an essential nutrient for mammals and for yeasts

In 1930 Norris and Ringrose[91] described in chicks a type of dermatitis which was eventually traced to deficiency of pantothenic acid In rats and foxes and probably other species deficiency of pantothenic acid causes greying of the hair Lipmann discovered that

this vitamin functions in metabolism as ' co-enzyme A (A for acetylating) Failure of acetylation in several metabolic steps causes profound physiological disturbance

(c) *Para-aminobenzoic acid* a substance which was long familiar to organic chemists was in 1940 found by Woods and Fildes[92] to be essential in bacterial metabolism The following year Ansbacher[93] reported that on an experimental diet containing pantothenic acid black rats turned grey Administration of *p*-aminobenzoic acid caused new growth of hair to be black Thus greying of hair was caused by either of two vitamin deficiencies

(d) *I inositol* was first isolated from muscle in 1850 by Scherer and was later found to be widely distributed in animal and plant tissues It was not until 1931 that it was found to be an essential nutrient for yeast and in 1940 Wooley[94] discovered it to be an essential nutrient for mice In inositol deficiency mice lose almost all their hair

(e) *Folic acid* Before the discovery of the role of copper in the utilization of iron for the synthesis of haemoglobin only those anaemias which responded to iron therapy were successfully treated Several types of anaemia among which pernicious anaemia had long been recognized did not yield to iron therapy In 1931 Lucy Wills[95] described a macrocytic anaemia in women in India which was alleviated by giving extract of yeast By restricting monkeys to a diet like that of the anaemic women she produced experimentally an anaemia like that of her patients Hogan and Parrott[96] using an experimental diet, produced a macrocytic anaemia in chicks which responded to liver extract. Several groups of investigators followed up these observations As a result a substance was isolated by Mitchell and his associates[97] from spinach which was essential for the growth of certain bacteria and to which they gave the name folic acid It proved to be effective for the relief of the macrocytic anaemia which results from the kind of deficient diet described by Wills It was also effective in treatment of the anaemia of sprue but did not alleviate all the symptoms of pernicious anaemia

(f) *Vitamin B_{12}* The discovery of this vitamin was due in large measure to the extensive studies directed to the isolation from liver of the factor or factors responsible for producing remissions in patients with pernicious anaemia Feeding large quantities of liver was first suggested by Whipple Minot and Murphy (1927)[98] studied the problem intensively and Cohn (1928)[99] prepared the first effective concentrate of the active fraction of liver In 1948 E. L. Smith[100] first isolated the active principle from liver In the same year Rickes and associates[101] succeeded in crystallizing a red substance which proved to be a cobalt-containing organic compound It was called B_{12} and proved to be effective in the alleviation of all the symptoms of pernicious anaemia.

Apart from its efficacy in the treatment of pernicious anaemia this vitamin functions in many specific ways in metabolic processes The great interest which its discovery aroused among nutrition investigators pathologists and clinicians is reflected in the publication within five years after its isolation of 1 134 scientific papers dealing with its physiologic roles

Vitamin B_{12} is able to bond certain other organic complexes in such a manner that there is a shifting of radicals the end result of which is synthesis of the sulphur-containing amino acid methionine Methionine is an indispensable amino acid and must be supplied by the diet unless certain micro-organisms which have the power to synthesize it when cobalt is present in the food are sufficiently abundant in the caecum or the large intestine of carnivores and omnivores The rumen bacteria of ruminants possess this power and inorganic compounds of cobalt suffice for their nutrition But in other kinds of animals and in man the intestinal florae fostered by many kinds of dietaries are of types which do not effect this synthesis and accordingly the complex organic cobalt substance B_{12} itself must be supplied Methionine is yielded in the digestion of many vegetable proteins in amounts below the nutritive needs of man and animals Its synthesis in the animal body by the catalytic property of B_{12} in its organized state in some macromolecule gives this vitamin a place of peculiar interest

The discovery of this property came about through the observation that certain diets of vegetable origin contained proteins of low biological value which could be greatly improved in their value for animal nutrition by either of two ways supplementing with an animal protein or the provision of vitamin B_{12} in the diet. Since vitamin B_{12} is effective in very minute amounts it could not be regarded as a direct source of the necessary amino acids It was evident that it must function indirectly to synthesize the needed amino acid or acids For a time it was known as a hypothetical substance called animal protein factor

After the discovery of folic acid and B_{12} and compounds of nutritive significance which may be derived from them or are related to them, the discovery of new vitamins apparently came to an end This is said notwithstanding the not infrequent announcement of a new factor which is believed by those who describe it to be essential for the prevention of some abnormality in a particular species

As with vitamins so also is there a likelihood that biological functions will be traced to elements not now believed to have significance in nutrition. In recent years it has apparently been established that molybdenum, and perhaps selenium, are essential

elements for animals Molybdenum is known to be essential for plant nutrition But search for evidence of the essential nature of inorganic elements other than those now known to have biological values and recognized as essential for the support of normal metabolism does not offer much promise of success

VI THE EVOLUTION OF DIETETICS

After 1900 Henry C Sherman became the most effective teacher of nutrition He was first to emphasize the importance of planning dietaries so as to provide not only enough protein and energy but enough iron and calcium also His studies showed that many people did not secure enough of these elements His text books on nutrition and on the chemistry of foods were widely used by teachers

In the years 1914–17 McCollum and his associates published their observations on the effects on young rats of feeding several kinds of seeds in combinations of two to five and in different proportions[99] Wheat oats maize peas beans millet and flax seed were studied No combination of seeds was found which enabled young rats to grow normally When a seed was combined with a suitable amount of alfalfa leaf flour the animals were far better nourished A seed supplemented with milk made a highly satisfactory diet They also found that young rats could grow well and remain in vigorous condition when fed milk alone and with tap-water to drink They also grew young rats to maturity and produced young on a diet of boiled egg yolks alone Similar experiments were conducted in which refined cereal products were fed with other foods to determine their supplementary values

They also determined the extent of utilization of the protein mixture in several seeds fed singly to young swine kept in metabolism cages Pigs weighing about [illegible] lb were fed only starch and water for 1 week and then were given only one kind of seed (corn wheat oats cooked peas or beans etc) After the animals had eaten only the food being tested during a 30-day period they were returned to a starch diet for another week During the entire experimental period the nitrogen intake and output were determined daily Then similar experiments were conducted in which a cereal was fed with several proportions of milk or other food The results clearly revealed good and poor combinations of foods Plant leaf and milk greatly improved the utilization of the proteins of seeds They also supplied sufficient calcium and vitamin A which McCollum s biological method for the analysis of a food had shown to be deficient in seeds These experiments emphasized the fact that singly individually some of our most widely used staple foods were deficient in certain nutrients and the importance of planning diets so as to include foods which made good each other s deficiencies

The new type of nutrition studies attracted sufficient attention to cause the Harvey Society to invite McCollum in 1917 to describe them in a lecture *The Supplementary Relations Among Our Common Food stuffs*[102] The following year he presented a more extended account of the new facts and viewpoints in the Cutter Lectures at Harvard University The latter were published in book form[103] Sherman and Lusk in new editions of their well known text books also gave the new ideas wide publicity After 1917 Osborne and Mendel turned from studying the nutrient properties of individual proteins combined with protein free milk and supplemented with individual amino acids to the study of the nutritive factors contained in different natural foods and extended knowledge in this field

Because of the outstanding values of milk and the leafy vegetables in those nutrients in which cereal foods peas beans potatoes and the commonly used root vegetables are deficient in varying degrees McCollum distinguished them as protective foods

In the lectures referred to above he pointed out that the typical American diet was derived to a great extent from refined wheat corn oats and rice among cereals and from muscle meats potatoes and sugar and that no combinations of these staple foods provided good or superior diets He urged dietary reform by the inclusion in the daily diets of more milk and leafy vegetables and reduction in sugar consumption Sugar being a purified substance which contained no protein vitamins or inorganic elements when consumed in considerable amounts crowded out of the daily menus other foods which were better constituted to promote health The effects of well planned and of poorly planned diets on experimental rats were widely dramatized by contrasting photographs

1 THE INFLUENCE OF THE HOOVER FOOD ADMINISTRATION ON DIETETICS

In the years of the First World War there was deficiency of foods in general in the European countries affected and wheat fats and sugar were in especially short supply in all the world This created a serious nutrition situation In April 1917 Herbert Hoover organized the Food Administration for the purpose of providing relief for undernourished people in warring countries Americans were asked to save wheat fats and sugar in order that foods might be sent abroad This required sudden change in meal planning based on the use of substitute foods for those commonly used in peace time People in America became more food conscious than ever before and

the patriotic spirit stirred them to make sacrifices from humanitarian motives Women were alert to follow the advice of those with special knowledge of foods, nutrition and dietetics The situation afforded excellent opportunity to teach people about good and poor combinations of foods The new principles of menu making then introduced have not been much altered since that period

The principles of dietetics included more and more for attention and emphasis as knowledge increased as the result of animal experimentation After the recognition of a fat soluble and a water soluble factor of unknown nature both of which were taken into the philosophy of meal planners in 1918 came consideration of the peculiar value of uncooked fruits and vegetables for the protection of the capillary blood vessels from fragility, long known but first emphasized by Hess in feeding of infants in 1915[104] and recommended for people of all ages in the publications of the U S Food Administration in 1918 Then followed the practical application of vitamin D to the prevention of rickets in infants and children after its discovery in 1922. With the steady advancement of knowledge of new dietary essentials consideration of the minimum requirements of each food factor and its distribution in foods was and is given to the feeding of the well and the sick Early in the Second World War the Basic Seven statements for guidance of housewives in the feeding of their families were formulated to simplify the problems of food purchasing and of menu planning to ensure adequacy of menus

2. The Discovery of Methods of Assay for Vitamins

The discovery of methods for quantitatively estimating the content of each vitamin in foods was of primary importance to dietetics These were of three types In some cases chemical methods were designed and are highly valuable, but in general these are less specific than those based on tests on living organisms. They involve determining the minimum amount of a vitamin which will promote growth or prevent or cure specific lesions caused by the vitamin deficiency *in the test animal In a number of cases micro*-organisms have been found useful because using an appropriate medium the amount of growth or other criteria in the life of the organisms depends on the amount of vitamin supplied in assay tests.

With quantitative methods available the amounts of each vitamin in fresh foods of many kinds have been determined Also the losses of these due to ageing, storage under different conditions canning, and *cooking by different methods are fairly accurately* known

3 Determination of the Amount of Each Nutrient for Optimum Health

Many attempts have been made to determine the minimum requirements of man and animals of several species for each of the nutrients which the diet must provide Requirements of rats and chicks are pretty accurately known but for humans from infancy through growth in pregnancy and lactation in infectious diseases and in the aged the values are not fully satisfactory One important consideration has been, and is the belief that there should always be a factor of safety over actual minimum requirements and on this point opinions of informed people have differed widely But minimum requirements have been agreed upon by qualified scientific groups Diet planning on the basis of assay values and nutritive needs now rests on a generally satisfactory basis

VII MALNUTRITION A NEW DIVISION OF PATHOLOGY

Clinicians and a few pathologists studied the nature and cause of beriberi before and after the discovery by Eijkman and Grijns that a condition analogous to the human disease could be brought about by restricting animals to polished rice as their sole food Their observations stimulated thought and experiment by others but since polished rice is deficient in so many essential nutrients the symptoms of the rice disease were those of multiple deficiencies and pathology was but little advanced by the investigations The experimental production of deficiency states referable to vitamins A and E and riboflavin respectively which were studied by the techniques of histology and pathology by Mori[illegible] Evans[illegible] and Wolbach[illegible] represented only the initial steps in the study of the pathology of these deficiency diseases. The discovery in 1922[illegible] that the newly discovered vitamin D prevented or cured experimental rickets in rats and that it was the substance to which cod liver oil owed its therapeutic value for rickets in infants and children, brought into view another specific state of malnutrition caused by lack of individual nutrients. These following as they did the ever growing knowledge and interest in the effects of depriving animals of one or more amino acids and of the profound metabolic disturbance *in bone growth* which resulted from unfavourable quantitative relations between calcium and phosphorus greatly stimulated the interest of chemists and pathologists in the study of metabolic disturbances arising from faulty diets.

Pathologists have contributed knowledge of the sequence of symptoms the initial and successive

morphological changes which become manifest when the diet of man or animals deviates to a significant degree from the optimum for maintenance of health Nutritional diseases have become recognizable by clinicians and remedial steps can be taken in the form of clinical dietetics A new branch of therapy has come into existence as the science of nutrition has advanced

XIII SOME CONTRIBUTIONS OF PHYSIOLOGICAL AND ORGANIC CHEMISTS TO NUTRITION RESEARCH

Physiological chemists not only conceived the idea of the promise of discovery afforded by sustained study of the causes of failure of experimental animals restricted to simplified diets composed of chemically characterized substances but achieved notable discoveries by isolating and describing each of the vitamins It was by the use of their methods that the physiological roles of the inorganic elements of biological significance came to light They alone had the understanding of the chemical basis of physiology and pathology and the vision of rewards for efforts to inquire into how each essential nutrient participates in the metabolic scheme They were led by the results of many experiments to conclude that both the vitamins and trace elements or micronutrients perform their most important functions in the nutrition of plants and animals by participating in catalytic functions in enzyme systems Certain of these determine the paths and steps in carbohydrate metabolism others play similar roles in fat and amino acid metabolism and include both anabolic and catabolic processes and so determine the metabolic characteristics of organisms having different enzyme patterns

Accordingly the development of methods for separating and in many cases for crystallizing enzymes and studying their action on suitable substrates has been rapid in recent years Physical methods for mechanically separating from cells the several histological elements such as nucleus mitochondria vacuoles and cell membranes make possible the examination of each to discover its complement of enzymes Such investigations together with others on intact organisms are revealing the secrets of body chemistry in health and the way it is disturbed in disease and in individuals whose diets deviate from the optimum in composition Understanding the chemical working of living things connotes discovery of techniques for preserving their integrity and harmony the realization of perfection of physiological well being The path to success in the management of one s life so as to promote optimum physical development prenatally and from birth to maturity the maximum attainable preservation of vitality in middle life and the deferment of the tissue changes accompanying senile deterioration lies in application of all available knowledge of foods in sound systems of dietetics

XIV SYNTHETIC VITAMINS AND OTHER NUTRIENTS AND THEIR ANALOGUES

High among the achievements of modern science stands the development of organic chemistry From apparently hopeless confusion and complexity in about 125 years scientists have discovered the secrets of the number and nature of the substances occurring in the plant and animal world and how their molecules are constructed how they may be resolved into simple derivatives and how the molecular architecture having been determined by well known techniques even large and complex molecules of many kinds can be synthesized This body of knowledge has been applied by organic chemists to the study of each of the vitamins as they were isolated from natural foods Their structures having been determined synthesis was soon achieved so that now most of them are available in large quantities and at relatively low cost In a few cases some micro-organism given the proper medium to grow in can synthesize a vitamin faster and more cheaply than chemists can in their glassware

An extremely important discovery was made possible by the synthesis of analogues and isomers of the vitamins These chemical relatives of the list of less than thirty organic nutrients which are peculiarly constructed to bond with each other to form macromolecules which aggregate in orderly associations to form cells of every specialized kind in the different tissues and organs and carry on the successive steps in metabolism rapidly and without error can act as impostor molecules in the building up of enzyme systems Their ungenuine composition is not detected when they are present among the essential nutrients absorbed in the body fluids and they may be bonded chemically in macromolecules in place of the uniquely constructed true nutrient but they cannot function in the metabolic process and so block it Hence they are called antimetabolites The pharmacological action of some drugs results from this property of blocking the metabolism of pathogenic micro-organisms This concept serves as a guide for organic chemists seeking to synthesize drugs useful for clinical purposes Antimetabolites offer the prospect, when the right

structures have been produced of being useful in the control of malignancies

1 Synthetic Vitamins Present New Nutrition Problems

The discovery of vitamins and of the fact that the milling of wheat and maize and the polishing of rice removed the vitamins from the portions used as human foods, brought refined cereals under unjust criticism as foods The great cereal producing areas in America Argentina and Australia are far from the principal centres of population which depend on them for bread stuffs There are sound reasons for milling white wheat flour and degerming corn meal which must be shipped long distances and undergo storage for considerable periods before they are consumed Rancidity and infestation with insects are the two most serious hazards to the marketing of whole wheat flour or whole corn meal All informed people know that there is no health hazard in the consumption of refined cereal products provided their nutritive deficiencies are supplied by other foods in the daily diet In perhaps half the world inclusion of sufficient milk and other dairy products and green vegetables accomplishes this The germs of wheat and maize which are milled out have gone into animal feeds at animal feed prices, but could easily be freed from fat to prevent rancidity and treated to destroy any insect eggs present In the defatted state they keep wholesome indefinitely and could be incorporated in bread to restore the full complement of water soluble vitamins The great quantity of yeast produced as a by product of fermentation industries might well be used as human food because of its richness in all vitamins except those soluble in fats These excellent vitamin resources have in great measure been neglected in favour of restoration of vitamins by incorporation of synthetic products

The temptation to people insufficiently educated about nutrition to misuse vitamins is very great because they hear such marvellous accounts of what they accomplish when properly used In full blown deficiency diseases the provision of the right vitamin causes spectacular recovery Less dramatic but highly gratifying are the responses of patients in states of early and mild deficiency of any vitamin They feel like new persons Patients with chronic wasting diseases who may develop secondary deficiencies have often shown marked improvement from vitamin therapy In pre and post-operative medical care and in patients with liver damage the administration of vitamins may be life saving However the profit motive has led to over-exploitation of vitamins They are recommended through every medium for gaining the attention of the public for such vague symptoms as weakness or fatiguability nervousness insomnia irritability gas indefinite digestive disturbances etc irrespective of the kind of diet being taken

Thus the medical profession scientists and teachers of dietetics find themselves in a dilemma In their efforts to instruct uninformed people they have to compete with graceless exploiters of credulity Those who are qualified to judge are in agreement that in the absence of organic disease the individual who employs a diet adequate in calories in which cereals are prominent but are supplemented with sufficient milk eggs meat fruits and green and yellow vegetables does not need and does not gain physiologically from taking additional vitamins

There can be no question about the usefulness of synthetic vitamins both in normal and clinical dietetics It is only meant here to emphasize the great temptation to misuse them in the interest of manufacturers

XV DIFFICULTIES IN EDUCATING PEOPLE IN NUTRITION

Since we possess such an imposing body of knowledge of the individual nutrients contained in our natural foods and the seriousness of deficiency of any one of them in the diet it would appear that there should be no difficulty about convincing everyone of the importance of right eating Sound teaching of dietetics to the public is confronted by serious difficulties. Health is universally acknowledged to be a precious possession and no one now doubts the importance of nutrition to health But teaching people how to plan good diets is not enough The practice of good principles of dietetics requires not only knowledge but a certain degree of conviction and of self-discipline Many people who accept sound teaching in principle are deficient in awareness of the importance of right eating and are but weakly motivated to alter established habits in order to realize the rewards which nutrition has to offer In the adolescent girl indulging the appetite often prevails over self-control for the reward of health beauty or forethought about future motherhood The middle aged may confidently be promised deferment of the signs of ageing as a recompense for prudence in eating habits Yet common observation shows that weak motivation generally prevails. Promise to the overweight of better comfort looks and longer life for refraining from some of the enjoyment of over-eating, stimulates the resolution of only a few The establishment of sound eating habits in childhood affords greatest promise of success in safeguarding health through the right use of foods.

XVI THE GROWING USE OF CHEMICALS IN FOODS AND FOOD PRODUCTION

With the development of modern industries an ever increasing portion of the populations of industrialized countries lives under city or suburban environments Their provisioning requires transport and storage of foods and care to prevent deterioration in appearance and nutritive values which is a new thing in human experience Food sanitation has assumed great impor tance in protection of the public health Scientific research in the canning industry has accomplished much in conserving desirable qualities in the foods which it processes as have likewise investigations relating to drying and freezing of foods These have important status in dietetics and are commendable but food technologists are in a questionable position in adding certain chemicals to a number of foods

Food processing industries use two general groups of chemicals One group adds essential nutrients to foods which contain too little of them either naturally or because of refining or sterilization Qualified officials are in a position to determine sound and unsound practices in this field

Another group of chemicals presents problems far more difficult to evaluate in their effect on health This includes chemicals which lend qualities desired by housewives and which are expected to increase sales Examples are eye appeal of fruits vegetables meats and fish fresh and frozen Other examples include additives to improve texture of bread and cakes to prevent fat from spattering in frying to retard staling of bread treatment of potatoes with chemicals to prevent sprouting hastening the colouring of fruits picked while unripe the use of antibiotics to prevent spoilage of fish etc

The increase in the use of insecticides and weed killers on farms and fumigation of cereals extends the possibility of contamination of foods with poisonous residues far beyond the long familiar residual con tamination of fruits with arsenic and lead from sprays used on trees Over all such grand scale use of poisons in agriculture must cause concern to all thinking persons because of the danger of food contamination

The growing use of antibiotics in animal feeding and of the recent practice of administering tranquil lizing drugs to increase rates of gain by animals for meat production must also be of concern until we have accumulated many more facts from experience The problems presented by these new techniques of dealing with agricultural handicaps are doubtless all soluble but they are new and it may well be that there is considerable blundering in early efforts to solve them The tremendous achievements of benefit to health to which scientific investigations have led greatly outweigh in importance the several types of hazards which food production and processing present all susceptible of solution by the scientific method

XVII IN PERSPECTIVE

On the occasion of the publication of the first volume of *World Review of Nutrition and Dietetics* it is appropriate to appraise what has been accomplished in these departments of knowledge and what the new knowledge promises for the future welfare of the human race In a period of about sixty years the effective use of animal experimentation brought to light the approximate number and the chemical nature of the forty-odd elements and organic compounds which are the primary nutrients for all living things except those capable of photosynthesis which can synthesize them all In the same period were discovered X rays radioactivity radium radio-telegraphy the techniques of aerial navigation atomic fission and the hydrogen bomb In the field of biology and medicine most of what we know of hormones amino acids vitamins the physiological significance of certain inorganic elements the sulpha drugs the antibiotics and the numerous synthetic drugs of importance in medicine was learned in the same period All these discoveries have profoundly affected human welfare for better or for worse

The discovery that each essential nutrient when in deficient supply causes a specific and unique type of metabolic disturbance by the symptoms of which it may be recognized in man or animals and the nature and occurrence in nature of each nutrient provided the means of prevention and cure of several diseases not hitherto recognized as being referable to dietary errors Seventy years ago beriberi scurvy in bottle fed infants rickets pellagra endemic goitre and perni cious anaemia were all great human scourges of which no one knew the causes and for which there were no methods of cure Today their aetiology is fully understood and all have been essentially eradi cated and will not again afflict any considerable number of people

If we were not in possession of the facts brought to light by animal experimentation many millions of people throughout the world who now enjoy health would be physically handicapped or incapacitated suffer pain and have short spans of life The special requirements of mothers during pregnancy for various nutrients are known and are in great measure provided ensuring health protection for mothers and improved prospects of infants for being physically well born The main problems of infant feeding have been solved Over large areas the incidence of dental caries has

been reduced by half That dietary factors are important in safeguarding the health of the blood vessels has been established and the kinds of dietary errors which lead to lesions of atherosclerosis are at present the subject of intensive study and should soon be understood

This newly gained body of facts is of universal benefit to mankind Its application represents the greatest achievement in the history of preventive medicine Dietetics ranks with immunization against contagious diseases sanitation and quarantine as a health measure

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2

Effects of Androgens and Oestrogens on the Metabolism of Proteins and the Growth of Tissues

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Contents

I INTRODUCTION

The sex hormones are known to produce an activation of the growth of genital organs and therefore they obviously have an anabolic influence on them But they also play a stimulating or inhibiting role in the development of other organs and tissues The result of these various actions reflects on the total nitrogen balance of the body

We shall try to examine the principal data on the metabolic action of androgens and oestrogens unfortunately our documentation is necessarily in complete because of the too extensive scope of the subject and limitations of space *

II THE ANDROGENS

The investigations on the metabolic action of andro gens have often had for their purpose to find a steroid showing a limited effect on accessory sex organs but a marked anabolic action on all other body tissues so that it could be used in certain pathological states with negative nitrogen balance

In fact it is now known that even the androgens which are most anabolic in their influence on nitrogen excretion exert a very different action on the various target tissues—some are stimulated others inhibited and still others are not influenced in their develop ment

The androgens like all other hormones interfere as activators or inhibitors with some enzymatic systems which in general take part simultaneously in several *metabolic processes such as the carbohydrate* fat and protein metabolisms or water and electrolyte excretion contributing thus to the elective development of some tissues and to the involution of some others Ultimately the total biochemical balances are often positive only to a slight extent and for a very short time

A. Effects on Growth and Maintenance of Body Weight in Mammals

In order to avoid repetition differences in growth between sexes will be reviewed in the part treating of the oestrogens here only the repercussions of orchi dectomy and the action of androgens will be analysed

1 Effects of Orchidectomy

In man prepubertal castration increases the statural growth because of the non-closure of epiphy seal centres The same phenomenon is observed in some domestic animals (ox sheep) On the contrary castration delays growth (either statural or ponderal) in the guinea pig (Ancel and Bouin 1906) and in the rat (Stotsenburg 1909 Donaldson and Hatai 1911 Tang 1941 Aschkenasy Lelu *1951) even if per* formed after puberty (Aschkenasy and Pariente 1952) However in rats deprived of alimentary proteins the fall in weight is not altered by castration (*ibid*)

2 Action of Androgens

(*a*) In the dog rat and mouse injections of testosterone propionate (TP) and other androgens provoke *a gain of weight which is more pronounced in cas* trated than in intact males unless the latter are in a state of exaggerated protein catabolism especially if hormonally induced—injections of ACTH (Selye 1955) or cortisone (Gaunt Tuthill Antonchak and Leathem 1953) If the catabolism is secondary to a dietary deficiency the beneficial effect of the androgens is much less pronounced being inhibited by the deficit in protein building material

The effect of androgens on weight is not accounted for entirely by the increase in alimentary intake Such an increase has been observed in castrated rats treated with these hormones but not in intact rats in which testosterone propionate (TP) rather reduces the appetite (Lelu 1943 Aschkenasy Lelu 1951)

In any case the weight gain caused by TP in male castrated rats is only temporary (Kochakian 1950) *persisting for no more than 10 to 15 days In the same* way in male rats recovering from a protein starva tion injections of TP accentuate the gain in weight only for a short time (Aschkenasy and Dray 1953–4)

The secondary exhaustion of the effect of androgens on somatic weight—a phenomenon to be found also for the nitrogen balance—shows that the general anabolic effect appears within the limits of a definite total dosage beyond this dosage the androgenic impregnation of the body induces some compensatory reactions

(*b*) In the young male pig, the oral administration of methyltestosterone does not exhibit any beneficial effect on growth and even retards it in the female (Noland and Burris 1950)

(*c*) In man stimulation of growth by the androgens has been reported in hypogonadic dwarfism and in some other delayed growths (Kenyon Knowlton and Sandiford 1944 Netter and Chevallier 1953) but no

* The survey of literature pertaining to this review was completed in February 1958

success has been pointed out in premature infants (Agerty and Seitchik 1952) Leanness from various causes has been treated successfully by the androgens for example by methylandrostenediol (MAD) (Albeaux-Fernet Robert and Berton 1952)

B Action on the Nitrogen Balance

1 Effects of Orchidectomy

This intervention has no uniform effect on nitrogen balance in the adult dog (Korenchevsky 1925) and rat (Lelu 1942*b*, 1943)

2 Action of Androgens

Kochakian and Murlin (1935) were the first to detect a decrease of urinary nitrogen in castrated dogs injected with an androgenic urinary extract One of us (Lelu 1942*a* *b*) observed the same effect after injection of TP in adult male rats on a protein free diet

Since then decreases in nitrogen excretion have been reported in male castrated animals after administration of various androgens by Kochakian (1946) and also by other workers (Braasch *et al* 1950 Kassenaar, 1953 Stafford Bowman and Olson 1954)

The magnitude of the action of androgens on nitrogen balance depends on the functional status of the gonads of the treated animal Thus intact male dogs and rats react less and later than castrated ones (Kochakian 1946 Kochakian Moe and Dolphin 1950)

On the other hand females are less sensitive than males Ovariectomy increases the efficiency of androgens which however are less active than in castrated males (Kochakian and Beall 1950)

The nitrogen retention produced by the male hormones is too important to be explained by the hypertrophy of the accessory sex organs alone and consequently must be attributed also to a stimulation *of the growth of other tissues The expression* ana bolic effect is often used to designate only this extra genital stimulation However like the weight gain the improvement of the nitrogen balance is only transitory (during the first two or three weeks) even if the hormonal treatment is continued The rapid exhaustion of the effects of androgens is not due to a definitive refractory state because a new transitory nitrogen retention is obtained if hormonal injections are given again after a rest period (Kochakian 1953)

In man temporary decrease in nitrogen excretion has been observed as the result of androgen treatment in hypogonadic states with or without dwarfism in thyrotoxicosis Cushing s syndrome in panhypopituitarism and Addison s disease in various types of cachexia and in alimentary deficiencies (Butler *et al* 1945)

Like that of the weight gain the magnitude of the nitrogen retention is influenced by the chemical structure of the androgen employed It is chiefly the *esterification of the steroids in position 17* by carboxylic acids especially by fatty acids of high molecular weight, which has been advocated for a long time (cf Junkmann 1957) to increase and extend the physiological effects of male hormones However many esters which have been synthesized in recent years have been assayed only for certain of their tissular actions, but not for their reducing effect on nitrogen excretion.

As to this latter action the propionic and acetic esters of testosterone were *considered in the beginning* as the most active But another ester recently tried, the cyclopentylpropionate (TCP) is endowed with a much more lasting activity in man (Lloyd and Fredericks 1951 Warren and Hayes 1952 McCullagh, McKendry and Schoffenburg, 1952 Howard Reifenstein Courtright and Turner 1953 Crispell Parson and Gahagan 1954) as well as in the animal (Ott, Kuizenga Lyster and Johnson 1952) A protracted nitrogen retention has also been observed with testosterone phenylacetate (TPA) (Howard *et al* 1953)

Implants of free testosterone are as efficient as the propionate but injections are less active 17 Methyl testosterone is active even *per os* (Talbot Butler and MacLachlan *1943) The 17 methyl derivatives of* androstane 3α 17β-diol (McSwiney and Prunty 1956) and of Δ_5 androstene 3β 17β-diol (MAD) (Talbot *et al* 1943 Wilkins and Fleischmann 1946 Homburger Kasdon and Fishman 1950 Querido Kassenaar Schuurs and Seldenrath 1952 McSwiney and Prunty 1956) show also some oral activity especially in man

Discordant results have been obtained with andro*stanediol and its diacetic ester* and also with Δ_5-androstene 3β 17β diol both much less active than their methylated derivatives

A significant nitrogen retention has been recorded *with various other steroids* recently synthesized, none of which however is more active than TP (Stafford *et al* 1954) These are—androstane-17β-ol 3-one (Pearson Weissberg and McGavack 1953) 6β-hydroxy 3 5-cycloandrostane 17-one (Kassenaar, Bekkum and Querido 1953) and above all 19 nortestosterone (Baker *et al* 1955) and its *cyclopentylpro*pionic ester which is much more anabolizing than MAD or androstanolone (Stafford *et al* 1954) and has a more protracted action than TP (Hollifield Crispell and Parson 1956)

Finally beneficial effects on nitrogen balance have been recently reported with 17 methyl and, especially 17-ethyl 19 nortestosterone (McSwiney and Prunty 1956 Spencer *et al* 1957 Aschkenasy 1958*b*)

All the above-mentioned steroids other than the testosterone esters are active not only by injection but also by mouth although much higher doses are

required with the latter route For some compounds such as MAD (Henderson and Weinberg 1951) oral administration has an effect on nitrogen balance in man alone but not in laboratory animals

Most of these compounds are not very androgenic In general their anabolic action has a preferential effect on perineal muscles and only considerable cumulative doses modify also the accessory genital glands

It is noticeable that whenever the androgens decrease nitrogen excretion they induce especially in man a retention of water inorganic phosphorus chloride, sodium and potassium

Thorn and Harrop (1937) were the first to detect a sodium and water retention in dogs treated with TP and with free testosterone the retention however was markedly less than with oestrogens

Similar effects combined with chloride potassium and phosphorus retention following TP injections have been demonstrated in man by Thorn and Engel (1938) and by Kenyon *et al* (1940) The cessation of the injections provokes a water chloride and sodium excretion whereas potassium is retained longer apparently because of its intra-cellular localization

A hydro mineral retention has been observed in man not only following TP but also after free testosterone implants and oral methyltestosterone androstanediol and its 17 methyl derivative (Williams *et al* 1945 Querido *et al* 1952)

The respective proportions of nitrogen water phosphorus and electrolytes retained in man under androgen treatment are similar to those present in various tissues especially muscular tissue This peculiarity has been found also in the rat for the ratio water/proteins by Korner and Young (1955) after repeated injections of MAD These authors demonstrated—as Kochakian *et al* (1950*b*) had done before —an increase of water and proteins in the whole body in opposition to a decrease of fat Probably the calories supplied by the combustion of fats are utilized for the synthesis of proteins activated by the androgens

The transitory changes of somatic weight induced by the androgens result from the opposite effects of these hormones on lipids on the one hand and on water and proteins on the other hand for this reason a strict parallelism does not exist between weight and nitrogen retention curves during androgenic treatments

C Comparative Actions on the Principal Fractions of the Excreted Nitrogen

The discovery of a temporary nitrogen retention caused by androgens led to the question which fraction of the excreted nitrogen was mostly influenced by this action Only fractions of the urinary nitrogen had to be considered the faecal excretion of nitrogen remaining unchanged after injections of androgens in the dog (Kochakian and Murlin 1935) and in the rat (Lelu 1943) as well as in man (Kochakian 1946)

(1) No noticeable change in the distribution of urinary nitrogen has been observed in the *orchidectomized* rat (Lelu 1942*b* 1943)

(2) The decrease in nitrogen excretion induced by androgens is especially related to urea (Kochakian and Murlin 1935, Lelu 1943) whereas it does not concern purine nitrogen (Lelu 1943)

The excretion of creatine is influenced in an opposite way by the androgens according as they are 17 methylated or not Non methylated androgens decrease creatinuria but this decrease as well as that observed in urea excretion is only temporary it is even followed by an increased excretion despite the continuation of the hormonal injections (Lelu 1943)

In man a retention of creatine has been observed in the course of androgen treatment in some cases of eunuchoidism and Addison s disease or of spontaneous creatinuria of children (cf Kochakian 1946)

The methylated androgens (especially methyltestosterone and methylandrostenediol or MAD) orally or parenterally administered increase the urinary excretion of creatine (Wilkins and Fleischmann 1945 Querido *et al* 1952) However this effect is not constant (Lagier 1953) and may be delayed (Querido *et al* 1952)

Because creatinuria is observed even with creatine free diets (Wilkins and Fleischmann 1945) the action of the methylated androgens seems to concern not only the excretion but also the synthesis of this substance The excessive urinary excretion might possibly be explained by the inability of muscular tissue to retain creatine formed in excess (Hoberman Sims and Engstrom 1948) The hypothesis has been put forward that the methylated androgens would play a role in the very synthesis of guanido-acetic acid and not only in its methylation (Sims 1949)

D Anatomical Distribution of the Metabolic Effects of Androgens

It is already known now that (i) only certain tissues benefit from the anabolic action of the androgens while many others are not influenced and still others are even injured by the action of these hormones (ii) the actions of the androgens which according to the kind of tissue are anabolic or catabolic are not at all as transitory as their action on the total nitrogen balance the return to a normal balance observed at a late stage of an androgen treatment actually results in the establishment of a state of equilibrium between the different types of metabolic effects

1 Organs and Tissues Which Are Favourably Influenced

These are essentially—(i) some of the cutaneous appendages the sensitivity of which has been studied especially in birds (ii) the accessory genital organs (iii) the perineal muscles (iv) the kidneys (v) the haematopoietic bone marrow (vi) the salivary glands

These various organs and tissues continuously receive benefit from the anabolic action of androgens for the complete duration of the treatment even if the latter lasts far longer than the transitory period of nitrogen retention and even if the animals are kept on a restricted diet In these latter conditions the growth of these tissues is only possible on account of the decomposition of some other tissues which supply the necessary protein materials

(a) *The Cutaneous Appendages*

The growth of the chick comb can by initiated merely by local application of TP (Schiller Benditt and Dorfman 1952) Greenberg *et al* (1948) have shown that in the capon receiving labelled methionine the androgens emphasize the incorporation of isotopic sulphur in the proteins of the comb

In the hen testosterone can induce a masculinization of the comb only after suppression of the antagonistic action of the oestrogens by ovariectomy (Brard and Benoit 1953)

In man and in other mammals male hormones stimulate the development and mitotic activity of the sebaceous glands (Ebling 1947, 1951, Lapière 1953) as well as of the epidermis itself (Eartly, Grad and Leblond, 1951, Bullough 1952) In addition the androgens control in man the topographic distribution of the hair (Hamilton 1942)

(b) *The Accessory Genital Organs*

It has been known for a long time that orchidectomy determines an atrophy of these organs (seminal vesicles prostate epididymis coagulating glands Cowper s glands, preputial glands penis) and that, on the contrary the administration of androgens increases their volume not only in castrated but also in normal males (Martins and Rocha e Silva 1929)

In females hypertrophy of the uterus (Korenchevsky and Hall 1937) has been observed under the effect of androgens, but only if the dosage was high enough to overcome the inhibition of gonadotrophic secretion (Korner and Young, 1955*b*) there is also an hypertrophy of the clitoris the vestigial prostatic tissue (Korenchevsky and Dennison, 1936 Homburger Forbes and Desjardins 1950) and the mammary glands (cf Mayer and Klein 1948)

The stimulation of the accessory genital organs in the male concerns chiefly the epithelial formations, while the oestrogens have an opposite effect (on the seminal vesicles and the prostate) atrophy of the epithelium and hypertrophy of the connective stroma. (*See* the chapter on the oestrogens.)

In the seminal vesicles, the androgens induce besides an hypertrophy of the tissue itself a retention of intracellular water (Rudolph and Samuels 1949). This phenomenon has been attributed to an accumulation of fructose and citric acid, the concentrations of which are decreased by castration and greatly increased by injections of androgens (Mann 1946 Mann and Parsons 1947 Humphrey and Mann, 1949 Levey and Szego 1955)

As in any other tissue the activation of protein synthesis is accompanied by an increase in nucleic acids which could be detected by an increased uptake of radio-active phosphorus (^{32}P) under the effect of testosterone (Fleischmann and Fleischmann 1952)

Slices of seminal vesicles, from rats treated with testosterone consume much more oxygen than slices from vesicles of castrated rats (Rudolph and Samuels 1949 Melampy and Porter 1952) This effect might possibly be linked with the activation of certain enzymes of the aerobic phase of intracellular glycolysis such as succinic dehydrogenase and cytochrome oxidase both of which are decreased following castration and increased after injection of testosterone propionate (Davis Meyer and McShan 1949)

In the prostate the androgens induce the same enzymatic disturbances of the aerobic glycolysis (Barron and Huggins 1944 Davis *et al* 1949)

The phosphatase activity of the seminal vesicles and of the prostate is also stimulated by the androgens acid and alkaline phosphatases in the monkey (Gutman and Gutman 1939) acid phosphatases in the dog (Pazos and Huggins 1945) alkaline phosphatases in the mouse (Atkinson, 1948) and in the rat (Dempsey Greep and Deane 1949)

The androgens also increase the transaminasic activity in the ventral prostate of the rat This activity possibly plays a role in the deamination of the free amino acids very abundant in this organ (Awapara, 1952) The concentration of these free amino acids decreases after castration (Marvin and Awapara, 1949)

Relations Between the Effects on the Accessory Genital Organs (Androgenic Effects) and the Chemical Structure of the Steroids (i) The androgenic activity depends partly on the nature of the group fixed at the 3-position of the steroid (Kochakian, 1949 1952) from this point of view the group *C=O (present in testosterone)* is superior to the group C—OH (androsterone, *iso*-androsterone or 17 methyl-Δ_5-androstene-3β 17β-diol (MAD))

(ii) The suppression of the 4-5 unsaturated linkage as in androstane-17β-ol 3-one, also diminishes the androgenic effects (Romani and Blum, 1957)

(iii) The same holds for the methylation in position 17 (Deanesly and Parkes 1933) as in methylandrostenediol (MAD) or methyltestosterone According to Lyster Lund and Stafford (1956) the androgenic as well as the myotrophic potency of the latter steroid could be enhanced by introducing an atom of fluorine at carbon 9 together with an oxygenation at carbon 11 (fluoro-hydroxy and fluoro-keto-methyltestosterone)

(iv) The androgenic activity may be further inhibited by the suppression of the 19 methyl group Indeed nortestosterone is 5 times (Holtkamp Heming and Mansor 1955) to 10 times (Hershberger Shipley and Meyer 1953) less androgenic than testosterone

(v) A decrease in the androgenic effects of testosterone has also been obtained by introducing an atom of chlorine at carbon 4 (4-chlorotestosterone) (Sala and Baldratti 1957)

(vi) Free testosterone is less active than its esters as we have already seen for its action on nitrogen balance This peculiarity could be imputed to its slower diffusion from the site of injection

Among the esters the β-cyclopentylpropionate is much more effective (in the castrated rat) on the seminal vesicles than the propionate (Sakamoto *et al* 1951 Ott *et al* 1952) The *iso*butyrate and the phenyl acetate also show in man a longer androgenic action than the propionate (Looney 1954)

The action of the various androgens is not always the same on the seminal vesicles and on the prostate

In the young normal rat the growth of the seminal vesicles exceeds that of the prostate Now whereas androsterone especially increases the latter organ testosterone and its acetic and propionic esters are principally active on the seminal vesicles (Deanesly and Parkes 1933) According to Ott *et al* (1952) the β-cyclopentylpropionate would best imitate the physiological stimulation induced by the normal testicular secretion

(c) *The Perineal Muscles*

Wainman and Shipounoff (1941) first pointed out an hypertrophy of the perineal muscles in guinea pigs injected with androgens Eisenberg and Gordan (1950) detected a similar action in the rat using as an index the weight response of levator ani (L A) They observed that steroids which determine a measurable nitrogen retention also stimulate this muscle so they proposed to use the ponderal increase of the L A as an index not only of the myotrophic action but also of the general protein anabolic potency of the androgens This potency is calculated on the ratio between the weight increases of the L.A and of the seminal vesicles (Eisenberg and Gordan 1950) The latter can be replaced by the prostate (Hershberger *et al* 1953)

In point of fact the significance of this test is far from being as general as has been alleged

The hypertrophy of the L A while not always related to the condition of the seminal vesicles and the prostate is more or less linked to the hypertrophy of the bulbo-cavernous muscle the ratio of the weights between these two muscles being around 3 or 4 to 1 (Aschkenasy 1958*a* *b*) The specific action of the androgens on these muscles seems to be related much more to their effect on the penis than to their action on the other muscles of the animal (Scow 1952 Aschkenasy 1958*a* *b*)

On the other hand in the male guinea pig the L A is but vestigial and the specific action of the androgens is directed especially on the retractor penis muscle (Kochakian Tillotson and Austin 1957)

At any rate even in the rat no parallelism can be detected between the weight of the L A and that of the other muscles or the extra genital organs including those which benefit from the anabolic action of the androgens such as the kidneys or the salivary glands (Aschkenasy, 1958*b*) (Table 2 1) Neither is there any correlation between the hypertrophy of the L A which persists for the whole course of the androgenic treatment and the decrease in nitrogen excretion which is only transitory

Action on the L.A and Chemical Structure of the Androgens Sakamoto Gordan and Eisenberg (1951) relying upon their L.A test demonstrated the superiority of the cyclopentylpropionic ester over the propionic ester of testosterone According to these authors MAD is as myotrophic as methyltestosterone even if at the same time much less androgenic (Gordan *et al*, 1951)

Some other androgens are also more active on the L A than on the accessory genital organs—19 nor testosterone (Holtkamp *et al* 1955) the cyclopentylpropionic (Hollifield *et al* 1956) and phenylpropionic (Overbeek and de Visser 1957) esters of 19 nortestosterone and finally the 17 methyl and 17-ethyl derivatives of the latter (Saunders and Drill 1956 Goldman Epstein and Kupperman 1957 Aschkenasy 1958*b*)

With the daily dose of 1 mg the latter steroid determines (by intramuscular injection) in the rat an hypertrophy of the L A which is twice as large as that obtained with the non substituted 19 nortestosterone (Saunders and Drill 1956)

On the other hand, 11 keto and 11 hydroxymethyl testosterone are three times more active on the L.A. than is methyltestosterone and their potency is still further intensified by introducing fluorine at carbon 9 (Lyster *et al* 1956)

Finally it has recently been reported that the acetate of 4-chlorotestosterone as well as the acetate and the cyclopentylpropionate of 4-chloro-19-nortestosterone also possess an important myotrophic action towards the L.A. (Sala and Baldratti 1957)

TABLE 2.1

Organ and Muscle Weights (Means in mg ± S.E.M per 100 g of Body Weight) in Castrated Control Rats and in Castrated Rats given by Mouth either Methyltestosterone or Ethylnortestosterone during 30 Days (5 mg per Day) Dry Weights in Parentheses

	Controls	Methyl testo-sterone	Ethyl nortesto-sterone		Controls	Methyl testo-sterone	Ethyl-nortesto-sterone
Heart	315±9	305±8	305±5	Seminal vesicles	18±2	25±2	25±3
Masseter	303±6 (74±1)	399±26 (99±7)	358±8 (89±2)	Prostate	8±2	15±4	13±4
Gastrocnemius	538±19 (140±6)	531±27 (141±7)	560±15 (147±3)	Penis	73±6 (22±2)	107±18 (32±5)	107±7 (36±4)
Bulbo-cavernosus	80±12 (22±4)	175±47 (45±12)	195±13 (49±3)	Liver	4004±84 (1152±24)	4271±112 (1256±27)	4201±295 (1312±25)
Levator ani	17±2 (7±1)	54±17 (17±4)	66±6 (17±1)	Spleen	160±22 (37±5)	165±18 (39±4)	161±18 (38±4)
Kidneys	756±17 (184±3)	779±28 (193±6)	779±21 (193±3)	Thymus	122±9	110±8	91±6
Urinary bladder	39±3	38±7	38±2	Submaxillary glands	162±8	168±6	179±7

As a matter of fact the action on the L A of almost all the above mentioned steroids is hardly superior to that of TP or free testosterone if all these hormones are administered parenterally at the same dose level These compounds are characterized not so much by the magnitude of their action on the perineal muscles but rather by the conservation of this action (even if they are administered by mouth) despite the relative weakness of their properly androgenic potency (action on the seminal vesicles and the prostate) However, this latter potency becomes more apparent if the doses are increased or the treatment prolonged (Gordan *et al* 1951 Partridge *et al* 1953 Korner and Young, 1955*b* as to MAD Aschkenasy 1958*b* as to ethylnortestosterone)

(d) *The Kidneys*

Since its discovery by Korenchevsky and Dennison (1934) the trophic action of the androgens on the kidneys has given rise to much research It is not observed in all species—it is entirely absent in the guinea pig and the hamster (Kochakian 1952) while it is particularly accentuated in the mouse

In the rat as well as in the mouse females are more sensitive than males (Korenchevsky and Ross 1940) but in both sexes the androgens show their effect especially under two conditions—(i) if the kidneys are in an involutional state after the removal of either the testes (Korenchevsky and Ross, 1940 Kochakian 1947 Scow 1952) or the hypophysis (according to Selye 1941 but not to Scow 1952) or following a total underfeeding (Quimby 1951) or a purely proteinic starvation (Aschkenasy 1953 Aschkenasy and Dray 1953–4) (ii) if a unilateral nephrectomy induces a compensatory hypertrophy of the remaining kidney (McKay 1940, Lattimer 1942 Halpern Cournot and Camu 1951)

However if the hormones are given in adequate doses and for a sufficient length of time a renal hypertrophy may be obtained even in intact rats on a normal diet (Korner and Young 1955*b* with MAD Aschkenasy 1958*b* with ethylnortestosterone)

The hypertrophy of the kidneys is related to a water retention together with an increase of the renal tissue itself (Pfeiffer Emmel and Gardner 1940)

The stimulation by androgens of the synthesis of renal proteins has been demonstrated by the increase of nucleic acids in kidneys after injections of TP in rats intoxicated by uranyl nitrate (Mandel *et al* 1953) and also by an increased incorporation of [^{14}C]-glycine in kidney slices of mice *treated with testosterone* propionate (Frieden Laby Bates and Layman 1957)

The androgens increase certain enzymatic activities of the renal tissue more particularly that of arginase especially in the mouse and the rat (Kochakian 1944 1945 Kochakian and Robertson, 1950) to a lesser degree in the guinea pig (Humm, Kochakian and Bartlett 1948)

This enzymatic activation depends more on the dose and the duration of the hormonal injections than on the degree of the renal hypertrophy and in any

case it persists far beyond the transitory period of nitrogen retention (Kochakian and Robertson 1950) Kochakian Reed and Erscheid (1954) also observe an increase in the aspartic glutamic transaminase in the kidney of the rat but not of the guinea pig

The anabolic action of androgens concerns not only the renal tissue but the entire urinary tract especially the urinary bladder which becomes atrophic after castration and enlarges after injection of free testosterone (Kochakian Endahl and Austin 1957) and TP (Aschkenasy 1958*a*) On the other hand methyl testosterone and ethylnortestosterone given by mouth are inactive (Aschkenasy 1958*b*) (Table 2 1)

Action on the Kidneys and Chemical Structure of the Androgens In castrated mice, Kochakian (1950*a*) found methyltestosterone *per os* both androgenic and nephrotrophic but in castrated rats the latter action is very weak after oral administration of 5 mg/day for 28 days (Aschkenasy 1958*b*) (Table 2 1)

Actually it seems that steroids hydroxylated at carbon 17 have a particularly high nephrotrophic potency while their androgenic action is moderate methylandrostane 17β-ol androstane 3α 17β diol and its 17 methyl derivative finally 17 methyl Δ_5 androstene 3β 17β-diol (MAD) (Kochakian 1946 Henderson and Weinberg 1951)

The nephrotrophic potency is parallel neither to the androgenic effects nor to the trophic action exerted on the perineal muscles Thus in male castrated rats receiving by mouth 5 mg of methyltestosterone or of ethylnortestosterone for 28 days the kidneys are only a little larger than in controls whereas with both steroids the perineal muscles are two or three times larger than in the non treated rats (Aschkenasy 1958*b*) (Table 2.1)

In fact the nephrotrophic action as well as the other effects of the androgens depends not only on the chemical structure of the steroids but also on the duration of the treatment and the amount of the dose Thus it is sufficient to increase the dose to produce an hypertrophy of the accessory sex glands even with steroids which are principally nephrotrophic at lower doses (Kochakian, 1952)

The renal hypertrophy lasts far beyond the transitory period of nitrogen retention (Kochakian Robertson and Bartlett 1950) thus it is obviously due to a redistribution of tissular proteins under the effect of the androgens and not only to the transitory action of these hormones on the nitrogen balance

(e) *The Haematopoietic Bone Marrow*

The erythrocyte counts are higher in the male sex in mammals and in man (cf Aschkenasy 1952) and orchidectomy often determines a slight hypochromic microcytic anaemia (Steinglass Gordon and Charipper 1941)

Conversely protracted administration of androgens (TP) increases erythrocyte counts and haemoglobin in normal guinea pigs (Larizza Notario and Castrola 1951) castrated rats (Steinglass Gordon and Charipper, 1941 Crafts 1946) and also in intact rats made anaemic by bleeding (Finkelstein Gordon and Charipper 1944) or by dietary protein deficiency (Aschkenasy 1954 Aschkenasy and Dray, 1953–4) (Fig 2 1)

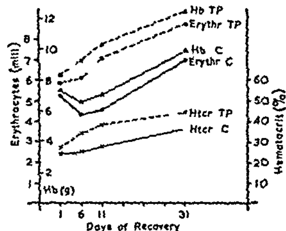


FIG 2 1 REPARATION OF ANAEMIA (ERYTHROCYTES HAEMOGLOBIN AND HAEMATOCRIT) IN RATS RECEIVING A REFECTION DIET POOR IN PROTEIN (7 PER CENT) AFTER 80 DAYS OF PROTEIN STARVATION

Comparison between rats injected with testosterone propionate (TP) and controls (C) injected with oil.

The stimulation of the erythropoiesis by androgens may be related in part to their interference with the utilization and blood transport of iron the sideraemia being subnormal in eunuchs and increased after an androgenic treatment (Lederer and Canivez, 1953)

The production of neutrophils by the bone marrow is also stimulated by androgens (Larizza *et al* 1951 Aschkenasy and Dray 1953–4) while on the contrary lymphopoiesis (Aschkenasy 1954) and especially eosinopoiesis (Aschkenasy and Dray 1953–4) are inhibited

(f) *The Salivary Glands*

The submaxillary glands are larger in male mice and rats than in females and castrated males (Lacassagne 1940 Bulliard and Delsuc 1941) Conversely injection of TP into intact male rats recovering from protein starvation accelerates the regeneration of these glands (Aschkenasy and Dray 1953–4) They increase also in male castrated rats maintained on a balanced diet and receiving either injections of TP (Fig 2.2) or ethylnortestosterone (Table 2.1) *per os* (Aschkenasy 1958*a* *b*)

In the salivary glands the protein synthesis is stimulated by androgens, especially in the tubular areas between the acini and the excretory ducts as indicated by the amounts of cytoplasmic ribonucleic acids which are more abundant in these areas in males than in females or in prepubertal animals (Junqueira *et al* 1949)

2 *Organs Injured by Androgens*

Androgens are especially harmful to the thymus

Castration in males is followed by an hypertrophy of the thymus (Chiodi 1938 Gregoire 1945) and less adrenalectomy but only if both operations are associated (Aschkenasy 1956)

The androgens determine a thymic involution (Laqueur, Hart and de Jongh 1926, Schacher Browne and Selye 1937 Scow 1952 Gaunt *et al.*, 1953) and exaggerate that following a protein deficiency (Aschkenasy 1953)

This thymolytic effect is most pronounced with testosterone esters (Fig 2 3) but can be obtained with any androgens even given by mouth, such as methyltestosterone or ethylnortestosterone if the treatment is sufficiently protracted (Aschkenasy 1958*b*)

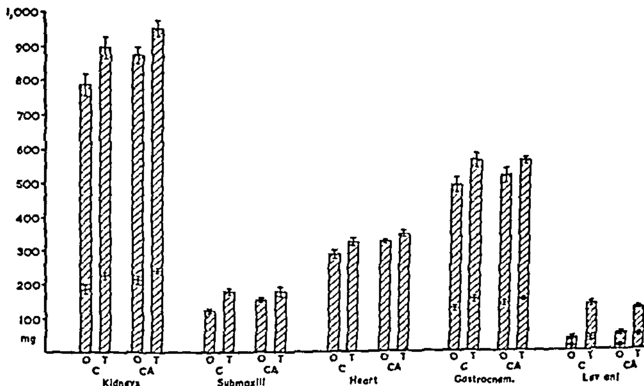


Fig 2.2. Relative Weights (S E.M) of Some Organs and Tissues in Castrated (C) and in Castrated-adrenalectomized (CA) Male Rats

O—Rats injected with olive oil T—Rats injected with testosterone propionate during 3 weeks. For the kidneys and the muscles gastrocnemius and levator ani the dry weights are also indicated

constantly of the spleen This hypertrophy has been observed even in castrated animals exposed to complete starvation (Szego and White 1951) or to a protein deficiency (Aschkenasy and Pariente 1952–3) *conditions which in intact animals induce an involution* of the lymphoid organs

Orchidectomy (in the rat) is also followed by an increase in the number of blood lymphocytes

In the course of a diet lacking in proteins, the deficiency lymphopenia is remarkably attenuated by orchidectomy (Aschkenasy and Pariente, 1952) However in some strains of rats hyperlymphocytosis does not appear after orchidectomy nor after

According to Reinhardt and Wainman (1942) the noxious action of testosterone affects in intact rats only the thymus and does not involve the lymph nodes and spleen except in castrated or thyroidectomized animals

However we did not detect any significant impairment of these two organs after 21 injections of 1 mg/day of TP in male castrated rats, whereas the thymus became atrophied TP induced a decrease of the spleen and sometimes *also a diminution of the lymph nodes*, only in rats both castrated and adrenalectomized (Aschkenasy 1958*a*) (Fig 2.3)

On the other hand, we succeeded in completely

inhibiting the regeneration of the thymus but not of the spleen by injecting with TP (1 mg/day for 30 days) intact male rats which previously had been deprived of dietary proteins for 60 days and which for the whole period of the injections received a repletion diet of a low protein content (casein 7 per cent) (Aschkenasy and Dray 1953–4)

However in rats maintained on a complete protein starvation injections of TP largely exaggerate the deficiency induced involution not only of the thymus but also of the spleen (Aschkenasy 1953) In these

may be inhibited by androgens (Gardner, 1947, Foley, 1950)

On the other hand it is already known that the development of metastases of breast cancer may be retarded in women by testosterone (Huggins 1952) or some other androgens such as MAD (Homburger, Kasdon and Fishman 1950)

To all these tumours more or less sensitive to the androgens may be opposed some others observed in man (cancer of prostate) which on the contrary are activated by these hormones (Huggins 1952)

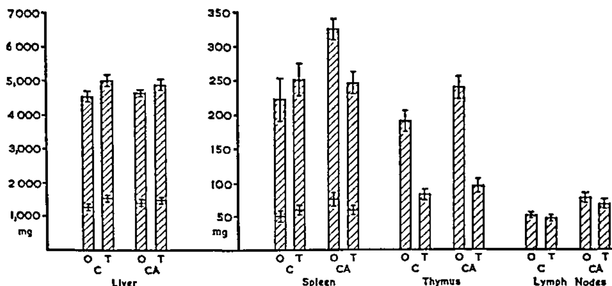


FIG 2.3 RELATIVE WEIGHTS OF THE LIVER (WET AND DRY) AND OF THE LYMPHOID ORGANS (WET AND DRY FOR THE SPLEEN) IN CASTRATED (C) AND IN CASTRATED-ADRENALECTOMIZED (CA) MALE RATS INJECTED WITH OLIVE OIL (O) OR WITH TESTOSTERONE PROPIONATE (T)

dietary conditions the spleen seems to contribute to the supply of the necessary nitrogen material for the neoformation of the accessory genital tissue which hypertrophies under the influence of TP even during protein starvation

Whenever the androgens decrease the weight of the spleen and lymph nodes they also lessen the number of the blood lymphocytes (Aschkenasy 1954)

Androgens show also a certain inhibiting action upon malignant hyperplasia of lymphoid tissue Thus orchidectomy increases the incidence of lymphoid leukaemia in susceptible strains of mice (Murphy 1944 Law 1947) and conversely testosterone prevents the development of lymphoid tumours in castrated mice (Murphy 1944) in mice treated with high doses of oestrogens (Gardner Dougherty and Williams 1944) and in mice made lymphomatous by X rays (Kaplan and Brown 1951) In point of fact not only malignant tumours of the lymphoid tissue but also experimental sarcoma and carcinoma of other tissues

3 ORGANS AND TISSUES OF WHICH THE SENSITIVITY TO ANDROGENS IS VARIABLE OR UNDER DISCUSSION

(a) *The Osseous System*

Young male rats castrated even after puberty generally show a delayed statural growth (Aschkenasy and Benhamou 1952)

On the other hand small doses of androgens accelerate the growth of bones in the rat (Rubinstein and Solomon 1941*b* Turner Lachmann and Hellbaum *1941—result discussed by Scow 1952)* and in the guinea pig (Coffman and Koch 1940) as well as in the hypogonadic man (Moricard and Bize 1937 Villaret *et al* 1938) In contrast large doses of androgens tend to inhibit the growth because of a premature ossification of the epiphyses (Deamer 1948 Sobel Raymond Quinn and Talbot 1956)

(b) *The Skeletal Muscles*

In man an hypertrophy of the skeletal muscles has been reported in a few cases of Leydig cell tumours

In the salivary glands the protein synthesis is stimulated by androgens especially in the tubular areas between the acini and the excretory ducts as indicated by the amounts of cytoplasmic ribonucleic acids which are more abundant in these areas in males than in females or in prepubertal animals (Junqueira *et al* 1949)

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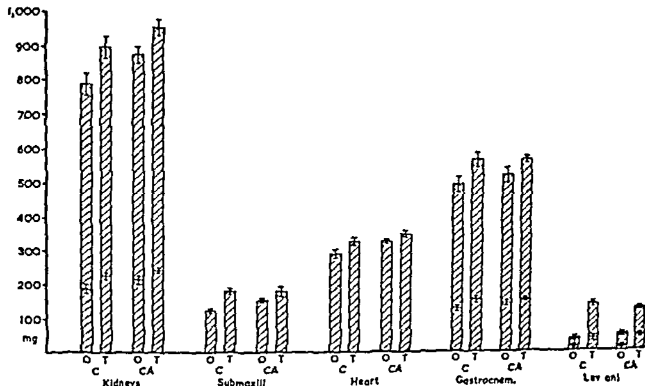


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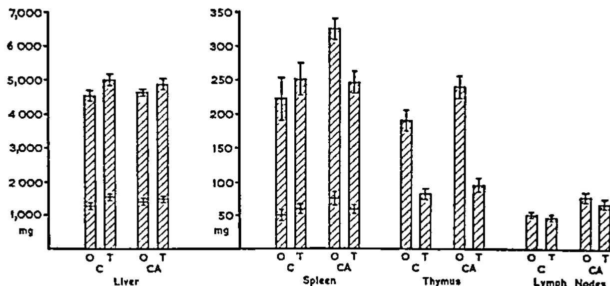


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(b) *The Skeletal Muscles*

In man, an hypertrophy of the skeletal muscles has been reported in a few cases of Leydig cell tumours

However in laboratory animals the myotrophic action of the androgens which have been synthesized until now affects only certain muscular groups

It is particularly pronounced in the guinea pig In this species orchidectomy is followed by a ponderal decrease not only of the perineal muscles but also of the head and neck muscles (Scow and Roe Jr 1953 Kochakian Tillotson Austin Dougherty Haag and Coalsen 1956*a*) Conversely the same muscles show a weight increase after androgen treatment (Papanicolaou and Falk 1938 Kochakian *et al* 1957)

Some other muscular groups are also sensitive to androgens in the guinea pig especially the muscles of the shoulder and the back diaphragm and caudofemoralis (Kochakian *et al* 1957) On the other hand the gastrocnemius is not influenced (Scow and Roe Jr 1953)

In the rat the myotrophic effects of the androgens are much less pronounced certain authors (Wainman and Shipounoff, 1941 Kochakian Tillotson and Endahl 1956*b*) affirm that neither orchidectomy nor androgens induces any significant changes in the skeletal muscles However according to our own experiments TP while being inactive in intact rats on an hypoprotidic diet (Aschkenasy and Dray 1953–4) induces a moderate hypertrophy of the gastrocnemius (Fig 2 2) and also of the masseter muscle if injected for 21 days (1 mg/day) into castrated rats on a well balanced diet

Nevertheless in the rat the sensitivity to the androgens is principally the property of the perineal muscles only certain particularly potent steroids such as testosterone esters have also an action on some skeletal muscles at least in castrated animals

(c) *The Heart*

Orchidectomy produces a decrease of the weight of the heart (Korenchevsky 1930 Korenchevsky and Dennison 1934) Conversely injections of TP enlarge this organ in castrated rats (Kochakian Reed and Eischeid 1954, Aschkenasy 1958*a*) (Fig 2 2) whereas methyltestosterone and ethylnortestosterone at least orally are devoid of any effect (Aschkenasy 1958*b*) (Table 2 1)

(d) *The Plasma Proteins*

In the female rabbit injections of TP increase proteinaemia the increase being related according to Sluczewski and Stora (1954) to the albumin fraction as well as to the globulins if the total injected doses are moderate and principally to the globulins if the doses are more important

In male castrated rats treated with TP at 1 mg/day for 21 days, the increase in the serum proteins affects particularly the electrophoretic fractions of the globulins (Aschkenasy 1958*c*) With lower doses of TP (1 25 mg three times weekly for 20 days) Leathem (1951) reports on the contrary an increase of the albumin/globulin ratio without changes in the total protein amounts

In man Homburger Daft Bonner Branche Kasdon and Fishman (1953) record an increase in the plasma proteins after treatment with MAD and Studnitz and Nyman (1957) call attention to the increase of α_2 globulins and especially of haptoglobin, in women treated with TP methyltestosterone or ethylnortestosterone

(e) *The Liver*

The action of androgens on the liver seems to depend on the amount of protein in the diet

If the diet is rich in protein or well balanced, no change is observed (Leathem 1951 Quimby 1951 Scow 1952 Tremolières Derache and Griffaton, 1955) or there is a moderate hypertrophy of this organ (Kochakian Robertson and Bartlett 1950 Aschkenasy 1958*a* *b*) (Table 2.1 and Fig 2 3) After orchidectomy the weight as well as the chemical composition of the liver are unchanged (Aschkenasy and Blanpin 1955)

On the other hand, on a protein free diet the deficiency induced atrophy of the liver is accentuated by TP injections (Aschkenasy 1953) It could be that in these conditions the liver proteins are transferred to the accessory sex organs and to the kidneys, which even if the dietary proteins are lacking, increase under the effect of the androgens

(f) *The Pancreas*

This gland is injured after TP treatment in the dog (Cornil Paillas and Rosanoff 1938) but not in the rat (Kerr Stears MacDougall and Haist, 1952)

(g) *The Testis*

In man as well as in intact animals a protracted androgen treatment induces a testicular atrophy (Moore and Price 1932, 1937 Gaunt *et al* 1952) with selective injury of the interstitial tissue because the gonadotrophic secretion of the hypophysis is inhibited However this atrophy may be prevented by increasing the doses of the androgens so that their direct stimulating action succeeds in compensating for the hypophyseal inhibition (Ludwig 1950)

For this reason a stimulation of the spermatogenesis by androgens has been observed chiefly in hypophysectomized animals (Walsh Cuyler and McCullagh, 1933 1937 Chu 1940 Eigler 1956) and also in hypophyseal deficiency states—in man (hypophyseal infantilism) (Moricard and Bize 1937 Warren and Hayes, 1952) as well as in animals for example in rats exposed to protein starvation (Aschkenasy 1953)

(h) *The Ovary*

Various androgens such as TP (Greep and Chester Jones 1950) MAD (Korner and Young 1955b) androstanolone and 19 nortestosterone (Payne Hellbaum and Owens 1956) decrease the weight of the ovary even in hypophysectomized animals

The action is therefore direct and does not depend on a gonadotrophic inhibition It corresponds exclusively to an atrophy of the follicles Moreover some androgens especially 19 nortestosterone are endowed with a direct progestational potency (Saunders Colton and Drill 1957)

(i) *The Thyroid*

Injections of TP do not modify the activity of the thyroid (Courrier Morel Zizine and Psychoyos 1956) which is an argument against the possibility of an hypothetical thyroid mediation in the anabolic action of the androgens

(j) *The Adrenals*

These glands are larger in the female than in the male rat and they hypertrophy after orchidectomy in the rat (Hall and Korenchevsky 1938 Aschkenasy and Pariente 1953) as well as in the guinea pig (Kochakian *et al* 1956a)

On the other hand the androgens induce in new born rats an adrenal atrophy with a selective involution of the zona glomerulosa (Selye 1940b) and they prevent the adrenal hypertrophy during some of the alarm reactions All these results might be explained by an inhibition of the ACTH production by the androgens (Zizine 1953)

However TP MAD and other androgens have been also reported to reduce the adrenal atrophy provoked by a protracted cortisone treatment or by hypophysectomy (Zizine 1953 Gaunt *et al* 1953 Winter Lollings and Stebbins 1953) On the other hand in male adult non-castrated rats repeated TP injections not only do not diminish but even increase the weight of the adrenals on a balanced diet (Money 1950) as well as during protein starvation (Aschkenasy 1953)

It is possible that the adrenal hypertrophy caused by castration is due to a compensatory overproduction of cortical androgens whereas the hypertrophy produced by androgens corresponds to a discharge of glucocorticoids induced by a direct action of these hormones

E. Factors Influencing the Metabolic Action of The Androgens

1 Routes of Administration

The androgens are active orally only at a much higher dose level than parenterally and the oral route in general is not sufficient to permit a significant nitrogen retention in the animals

The intravenous (Brown and Samuels 1956) and intraperitoneal (Bernstorf 1957) routes are also less favourable than the subcutaneous and intramuscular ones

2 Receptivity of the Tissues

This is in a large way influenced by the age of the animals Thus the accessory genital organs are particularly sensitive to the androgens during the period of puberty (Hooker 1942 Korner and Young 1955)

Hibernation prevents the hypertrophy of the seminal vesicles in castrated hamsters injected with TP (Lyman and Dempsey 1951)

3 Dietary Factors

(a) *Protein Levels in the Diet*

Some skeletal muscles (masseter and levator scapulae) hypertrophy during androgen treatment only in normally fed guinea pigs but not in fasting ones (Kochakian Tillotson and Austin 1957) In contrast the anabolic effects of the androgens on the accessory sex organs and on the kidneys appear even in the absence of any exogenous protein intake (Aschkenasy 1953) In these dietary conditions the transferred proteins come chiefly from the lymphoid organs and the liver organs which undergo a very important involution in protein deficient rats treated with androgens

(b) *Folic Acid Levels in the Diet*

In contrast to what has been reported for oestrogens (Hertz 1945) a deficiency in folic acid (obtained either by a diet deprived of this vitamin or by the use of an antagonist) by no means prevents the anabolic actions of the androgens in male animals—growth of the comb in cockerels (Zarrow Korefsky and Zarrow 1951) or development of the seminal vesicles in rats (Kline and Dorfman 1951) Nevertheless administration of folic acid increases the androgenic action of testosterone (Penhos 1954)

On the other hand folic deficiency inhibits the stimulating action of testosterone on the oviduct of the chick (Kline and Dorfman 1951) just as it inhibits a similar action of the oestrogens Perhaps this peculiarity is due to a specific need of folic acid for the growth of oviductal tissue or to the fact that the action of testosterone upon this organ depends on its previous conversion into an oestrogen (Kline and Dorfman 1951)

4 Hormonal Factors

The question has arisen whether the metabolic effects of the androgens are direct and independent or whether they need the presence of other hormones for their manifestation.

(a) *Nitrogen Retention and Weight Increase*

The temporary nitrogen retention and the weight increase induced by androgens have been ascribed to a stimulation of the somatotropic secretion (Shay *et al* 1941) but both effects may be observed in hypophysectomized rats (Kochakian 1950*b* Scow 1952 Rupp and Paschkis 1953, Korner and Young 1955)

The action of the androgens (TP) on the nitrogen balance has also been recorded in adrenalectomized rats (Kochakian Moe and Dolphin 1950 Kassenaar 1953 Korner and Young 1955) as well as in rats which have been made diabetic with phlorizin or alloxan (Wright and Kochakian 1953) or hypothyroid after thyroidectomy (Rupp and Paschkis 1954) or by thiouracil (Kochakian and Dolphin 1955)

Consequently neither adrenals nor pancreas nor thyroid seem essential to the protein anabolic action of the androgens However the thyroid and adrenal hormones and also some of the hypophyseal hormones play a certain role in the anatomical distribution of the metabolic effects of male hormones

(b) *Action on the Organs and Tissues*

(i) *Role of the Hypophysis* The prostatic atrophy after hypophysectomy is more important than after orchidectomy in the dog (Huggins and Russell 1946) This may perhaps be related to the suppression of the secretion of prolactin Indeed injection of this hormone magnifies the response of the accessory genital glands to testosterone propionate (Chase Geschwind and Bern 1957)

On the other hand according to Scow (1952) testosterone propionate fails to induce any increase in the size of the kidneys in hypophysectomized rats

(ii) *Role of the Thyroid* The presence of this gland seems to be necessary to permit the action of testosterone on the submaxillary glands of the castrated rat since this action is missing in the thyroidectomized animal and appears again after thyroxine treatment (Grad and Leblond 1949) Similarly TP does not induce any renal hypertrophy in thyroidectomized rats (Scow 1952 Kochakian Reed and Eischeid 1954)

On the other hand no synergy has been detected between thyroid and testicular hormones for any other specific actions such as that of the androgens on the seminal vesicles or the levator ani muscle (Scow 1952)

Concerning some of their tissular actions testosterone and thyroxine are even antagonistic—action on the epidermis (Eartly Grad and Leblond, 1951) or on the lymphoid organs (Aschkenasy 1953)

(iii) *Role of the Adrenals* While they are not essential to the action of the androgens on the nitrogen balance since this action has been detected in adrenalectomized animals as well as in patients suffering from Addison's disease (Thorn and Engel, 1938 Talbot *et al* 1943) the adrenal hormones still interfere in the tissular distribution of the metabolic effects of the male hormones.

This becomes evident from an experiment performed in our laboratory on the effects of TP on various organs and tissues of male castrated rats and of rats both castrated and adrenalectomized (Aschkenasy 1958*a* *c*) In each group animals treated with TP were compared to controls injected with olive oil (Fig 2.2 and 2.3)

While the increase in the relative weight of the accessory genital glands was similar in both groups, several extra genital anabolic effects of TP were less marked in the castrated-adrenalectomized group than in the castrated group—for example the relative hypertrophy of the liver kidneys submaxillary glands heart, gastrocnemius muscle and also the perineal muscles

On the other hand adrenalectomy exaggerated the lympholytic effects of TP while the contrary would rather have been expected—indeed the reduction of the thymus was more important in the absence of the adrenals the spleen and less constantly the lymph nodes decreased under the effect of TP only in castrated-adrenalectomized rats but not in rats simply castrated (Fig 2 3)

Finally the removal of the adrenals also modified the effects of TP on the serum proteins chiefly by amplifying the beneficial action of this hormone on the albumin fraction

The dietary intake being not very different in both groups of rats the decrease in certain extra genital anabolic effects of TP does not seem to be due to a decrease in the appetite induced by adrenalectomy It should be attributed rather to a defect in the degradation of tissue proteins used as building material for the tissues selectively stimulated by the androgens. In fact it is known that adrenalectomy is followed by a decrease of free amino acids in the blood (Ingle Prestrud and Nezamis, 1948)

It is nevertheless remarkable that the noxious effect of TP on the lymphoid organs has not been prevented but on the contrary increased by adrenalectomy This lymphoid involution was perhaps responsible for the decrease in γ globulins observed in several castrated-adrenalectomized rats injected with TP Such a parallelism between the two effects has been also observed in rats injected with ACTH cortisone and prednisone (Aschkenasy 1957)

F Enzymatic Mechanisms Involved in the Metabolic Action of the Androgens

The tissular action of the androgens involves some changes in certain enzymatic activities. However these changes are different according to the tissues and the animal species none of them may be considered as typical of the action of the androgens.

Thus in the rat and the guinea pig the acid phosphatase is increased in the accessory genital glands (Dempsey, Greep and Deane, 1949) but not in the kidneys (Kochakian and Vail, 1947) nor in the liver (Humm, Kochakian and Bartlett, 1948) nor in the blood serum (Buchwald and Hudson, 1944).

On the other hand, arginase is increased in the kidneys of the rat and the mouse while it is not much influenced in the guinea pig and even decreased in the hamster (cf. Humm *et al.*, 1948). In the rat, the increase in the renal arginase contrasts with the lack of change in the liver arginase (Kochakian, 1946) and the decrease of the same enzyme in the submaxillary glands (Kochakian, Endahl and Hull, 1955). Besides, the increase in the renal arginase activity is produced not only by androgens but also by cortisone. Furthermore, this increase continues in the course of a treatment with androgens even after the effect of these hormones on the urinary nitrogen has been exhausted (Kochakian and Robertson, 1950).

Consequently, arginase does not contribute to the general anabolic action, purely transitory, of the androgens but only plays a role in the action of these hormones on the renal tissue.

The increase of the transaminases also produced by the androgens seems to have a more general significance than that of the arginase. Indeed, it has been found in the heart and the kidney (Kochakian, Reed and Eischeid, 1954) as well as in the ventral prostate and the seminal vesicles of the rat (Awapara, 1952) and also in the temporal muscle of the guinea pig (Kochakian and Endahl, 1956).

G. Conclusion

The androgens are not endowed with a general anabolic potency on the tissue proteins as one might naturally conclude from their favourable transitory effect on the nitrogen balance.

Actually, their action is intricate—anabolic on various tissues (accessory genital tissue, kidneys, salivary glands, haematopoietic marrow and some muscles) but catabolic on the thymus and variable on some other tissues, for example on the liver, as well as on several skeletal muscles, this at least in the rat.

Between the tissues which are favourably influenced there is an hierarchy depending on the duration of the hormonal action and the chemical structure of the androgens. The tissular effects of these hormones are also influenced by the protein content of the diet and result in part from a synergistic action of the hypophyseal, adrenal and thyroid hormones.

The enzymatic anomalies induced by androgens, as far as they are presently known, do not explain in a satisfactory manner the metabolic effects of these hormones.

III. THE OESTROGENS

A. Action on Growth and Maintenance of Body Weight. Role of the Appetite

1. Differences Between the Sexes

The statural and ponderal differences in relation to sex are not purely genetic—they are emphasized at the time of puberty.

(a) *Rat*

In 28-day-old rats, the frequency diagrams of weight of animals having a similar hereditary and nutritional history are identical in both sexes (Sherman, 1952). Growth curves, hardly different at the beginning, remain parallel until puberty. From the fortieth or the fiftieth day, growth is slower in the female than in the male.

In the female rat, the oestrus is accompanied by a loss of weight together with a decrease in the dietary intake (Brobeck, Wheatland and Strominger, 1947). The difference of the appetite, which will be reviewed later, is certainly one of the factors interfering in the divergent growth pattern of the sexes. On the one hand the female eats less than the male; on the other hand, a poorer utilization of food is related to the presence of female hormones. In young litter mate rats force fed for 6 weeks with an equal quantity of the same isocaloric diet, Kim, Magee and Ivy (1952) observed that females grow less than males.

(b) *Human Species*

In general, girls remain shorter and thinner than boys; this could be due, according to Talbot and Sobel (1947), to the enhancing effect of androgens on the growth of boys. Nevertheless, according to the same authors, an ovarian deficiency which delays the skeletal and sexual maturation in adolescent girls does not cause their growth curve to deviate from normal.

In the human species, where various extra physiological factors influence the dietary intake, it is difficult to correlate the sex differences in growth with differences in appetite. Nevertheless, it is known that treatments with high doses of oestrogens provoke a severe anorexia.

2. Effects of Ovariectomy

The effects of gonadectomy in the female result not only from the suppression of oestrogens but also from

that of progesterone It seems, however, that the effects of the lack of oestrogens prevail

(a) *Rat*

According to Korenchevsky (cf Terroine 1952) gonadectomy sometimes produces thin and some times fat and heavy castrates in either sex It is now established that in the rat gonadectomy in duces opposite effects in the male as compared to the female

In well fed animals castration performed before puberty slows the growth of the male, while it acceler ates that of the female (Holt Keeton and Vennesland 1936 Lanson Golden and Severinghaus 1939 Tang 1941) There is true growth and not only fat deposition because there is an increase in the statural as well as in the ponderal growth (Holt *et al* 1936) and the tibias are longer in castrated female rats than in controls (Tang 1941) Furthermore the lipid content of the ovariectomized females is not above that of normal female rats (Holt *et al* 1936)

The contradictory results obtained by various authors may be explained by the previous nutritional status of the subjects under study We observed that in a strain of under-developed rats, not attaining at 30 days of age the usual average weight of our colony the castration of the males enhanced their growth, in contrast to what happened in healthy animals

We shall see later that oestrogens inhibit the appetite and that it is undoubtedly one cause of their depressive action on growth

It could be questioned conversely whether the stimulation of the growth after ovariectomy results from an increase of the alimentary intake Such an increase has been reported by Holt *et al* (1936) while it does not appear after orchidectomy According to Tang (1941) gonadectomy is followed by a decrease in appetite in both sexes but the differences pointed out are smaller for the female sex than for the male Aschkenasy Lelu (1951 1958) observed that the females castrated before puberty eat as much as and often more than their normal controls the increase involves the non protein components of the diet and not the protein consumption which does not vary Ovariectomy results in a greatly reduced spontaneous activity of the females (Wang 1923 Richter 1927) and their energy expenditure is thus at a lower level However they eat as much as if not more than their intact controls This energy imbalance can be com pared to that observed by Brobeck *et al* (1947) in an opposite direction in the normal sexual cycle of the female rat—there is hyperactivity during oestrus, but nevertheless a decrease in the dietary intake which explains the weight loss observed during this phase of the cycle

(b) *Human Species*

Ovariectomy as well as physiologic menopause, generally induces an increase of adiposity and weight. These conditions seem to be associated with a dietary intake above the energy requirements

The homeostatic imbalance so induced is often attributed by the clinicians to a psychic compensatory phenomenon, while actually it is perhaps due to the *suppression of the physiological inhibition* exerted by the oestrogens on the appetite

3 Action of Oestrogens

(a) *Rodents*

Since Spencer Gustavson and D Amour (1931) first observed inhibited growth in young rats treated with an hormonal extract from the urine of a gravid female a delayed growth in the young animals and a weight loss in male or female adults have been often reported during treatment with natural or synthetic oestrogens The conclusions have yet to be better defined As a matter of fact Baker and Everett (1944) point out that small doses of diethylstilboestrol (DES) increase the body weight of the immature young animals but high doses induce a weight loss in the adults either obese or not (Ingle 1948) Kochakian (1947) also concludes that the action of oestrogens on the growth of rodents is related to the dose—implants of natural oestrogens increase the body weight of male castrated mice at lower doses while they inhibit the growth and decrease the weight at higher levels In another study Kochakian Garber and Bartlett (1948) report a marked growth inhibition in the male castrated mouse under the effect of implanted *α-oestradiol or methoxy bis-dehydro-doisynolic* acid (MDDA) This inhibition furthermore affects also the linear growth (Gaarenstroom and Levie 1939 Bourlière and Gourevitch 1949 Hartsook and Magruder 1956)

The possible influence of age on the growth effect of oestrogens has been reported (Noble 1939 Baker and Everett 1944 Bourlière and Gourevitch 1949) —*under 80–100 g the young rat is not affected* by the inhibitory action of these hormones

The effect of the oestrogens on growth could also be related to the nutritional status of the animals One of us (Lelu 1943) has seen that female rats on a protein-deficient diet hardly modify their curve of weight loss while injected with moderate or high doses of oestradiol benzoate But according to Aschkenasy and Dray (1954) a weight gain above that of controls is observed in rats recovering from a protein deficiency and receiving very small doses of oestradiol benzoate

This discrepancy in the results seems to be related to the age of the animals, their nutritional status and especially the doses used. In most cases high doses of

oestrogens inhibit the growth of the rat inducing true dwarfism in the young

This property was at first attributed to a disturbed secretion of the hypophyseal growth hormone (Zondek 1936 Gaarenstroom and Levie 1939 Griffiths and Young 1942) But Zondek (1936) has pointed out that the hypophysis of treated animals contains as much growth hormone as that of controls Thus not the synthesis of this hormone but its excretion into the blood stream might be disturbed

This delayed growth may in fact be due solely to the reduction of the dietary intake provoked by oestrogens (Korenchevsky and Dennison 1934 Ingle 1941 Lelu 1943 Blanchard and Stebbins 1945 Cameron Guthrie and Carmichael 1946) Indeed rats treated with oestrogens but forcibly fed show a normal growth (Ingle 1941 Ingle *et al* 1947) According to Meites (1949) the growth of a female rat receiving no more food than is spontaneously eaten by a corresponding animal treated with DES (0 001 0 01 or 0 1 mg per day) does not exceed the growth of the latter The highest dose induces the strongest inhibition However Meites (1949) remarks that natural oestrogens could slow growth without influencing appetite Glasser (1954) suggests also a direct effect of oestrogens on growth for the weight loss is significantly higher in rats treated with oestrogens than in their pair fed controls

The reduction of intake appears without delay and very drastically but it vanishes if the treatment is protracted In addition there is a concomitant decrease in the water intake (Noble 1939 Meites 1949)

By means of a suitable experimental design Aschkenasy Lelu (1951 1958) has shown that the decrease in dietary intake concerns only the non protein components of the diet but not the protein part the ingestion of which remains the same and even becomes larger if the treatment is protracted Therefore it must be admitted that besides a decrease in the total intake there is a diminished efficiency per gramme of ingested nitrogen under the effect of the oestrogens

Thus oestrogens seem to disturb the quantitative adjustment of alimentary intake to energy requirements This effect may be related to the diencephalic action of these hormones (Brobeck *et al* 1947) Degeneration of the supra-optic nucleus has been reported by Weil and Zondek (1939) and Vasquez Lopez (1952) According to Stutinsky (1953) high doses of DES increase the neuro-secretion of the hypothalamus at the same time as the secretion of the antidiuretic hormone

It could be supposed that oestrogens act in the opposite way to a compound like gold thioglucose the effects of which are similar to those obtained by the destruction of definite hypothalamic areas (Waxler and Brecher 1950) Thus these hormones would stimulate the ventromedial area of the hypothalamus the excitation of which decreases the intake Or else they might injure the lateral area the destruction of which has the same unfavourable effect on the intake

(b) *Domestic Animals*

The problem of the action of oestrogens on growth has not the same significance in domestic animals where practical considerations prevail

The breeders use more and more oestrogen treatment in order to improve the growth of the animals or at least the yield in live weight In fact the use of oestrogens for this purpose is connected with the ancestral custom of castrating domestic animals particularly males in order to favour the fattening The injurious action of oestrogens on testicular function leads actually to a real hormonal castration easily obtained by the implant technique and by orally active synthetic oestrogens

(i) *Birds* The hormonal castration by oestrogens is performed on an industrial scale in some countries on the cockerel turkey and duck There is improvement not only in the quality of the meat but also in the yield in live weight We shall see later whether this favourable action on weight is concerned with the protein or lipid metabolism or with water retention. Bird (1946) affirms that high doses of DES increase the dietary intake of the chicken

(ii) *Domestic Mammals* Many authors assert that cattle react to treatment with oestrogens by a stimulation of growth in any case by a weight increase and an improved food efficiency These effects have been noticed in the steer (Clegg and Cole 1954 Burroughs *et al* 1954) and in the sheep (Struempler and Burroughs 1956) In the pig however no improvement of growth or alimentary efficiency has been observed (Peery Beeson and Andrews 1954 Taylor *et al* 1955 Sewell Warren and O Mary 1957)

The authors who remark an improvement in the growth generally note at the same time a stimulation of the appetite However an inhibition of the latter has been reported in the sheep (Bennetts 1946 Austin *et al* 1947) and in the goat (Meites and Turner 1948)

B Action on the Nitrogen Balance

Only studies on urinary nitrogen excretion made it possible to decide whether the changes of body weight under the effect of oestrogens really reflect changes in protein synthesis or are due only to an accumulation of lipids or water

stimulation or a particular injury of some tissues or organs? To consider this question it will be necessary to distinguish between the case of the mammals and that of the birds because the same organs do not react in a similar manner in the two groups

1 Mammals

(a) Organs and Tissues Selectively Stimulated

Oestrogens control the growth of the endometrium and myometrium of the uterus these stimulating properties being used as a specific test for the action of these hormones Certain accessory genital organs are also stimulated

(i) *The Uterus* The hypertrophy of the uterus of castrated rats treated with these hormones is chiefly related to a marked water retention Furthermore the oestrogenic stimulation increases the water content of many other tissues outside the genital area (Zuckerman Palmer and Bourne 1939) This initial oedematous reaction is followed by growth of the uterine tissue itself (stroma and epithelium) testified by an increase in the protein nitrogen and in the nucleic acids (Davis Meyer and McShan 1956) The alkaline phosphatasic activity increases (Atkinson and Elftman 1946 Jeener 1947) at the same time as the ribonucleic acid content (Jeener 1947 Borell 1952 Davis *et al* 1956) Roche and Nataf (1953 1954) noticed that the arginasic activity absent in the uterus of castrated guinea pigs appears after oestradiol treatment Noall *et al* (1957) injected young female rats both with oestradiol and a tracer dose of a labelled metabolically inactive amino acid amino-*iso*butyric acid Twenty hours later the uterus exhibited an accelerated growth and incorporated the amino acid up to 280 per cent of its initial value while there was no increase of incorporation into the liver

(ii) *The Mammary Gland* Oestrogens are responsible for the development of the excretory system whereas progesterone stimulates the growth of the glandular acini Nevertheless the enzymatic changes found up to the present in oestrogen treated animals concern more the glandular acini than the excretory canaliculi (Folley and Greenbaum 1947)

The anabolic properties of the oestrogens on the sexual effectors of females may furthermore, favour pathological hyperplasia in these organs (uterus mammary gland) Thus these hormones have been considered to be partly responsible in the genesis of some genital and mammary carcinomas of woman.

(iii) *The Pubic Symphysis* The connective tissue of the symphysis shows an important development under the effect of oestrogens, before the relaxation of the pelvic ligaments and the separation of the pubic bones. Roche Nataf and Marois (1950) and Nataf (1953) notice a parallelism, in the guinea pig, between the alkaline phosphatasic activity of the ligament and the degree of development of this tissue expressed in protein nitrogen

(iv) *The Vagina* There is a marked proliferation and keratinization of the epithelial cells under the action of oestrogens These changes are accompanied, according to Jeener (1947) by a large increase in the alkaline phosphatasic activity in the cytoplasm of the cells This fact has been confirmed by determinations of the alkaline phosphatasic activity of vaginal extracts At the same time, the cytoplasmic ribonucleic acid increases considerably (Kamell and Atkinson, 1948)

(v) *The Skin* Bullough (1952) points out the mitogenetic potency of the oestrogens on the epidermis of the adult mouse

(vi) *The Osseous Tissue* Oestrogens provoke osseous neoformation in the medullary canal of the bone This neoformation involves the appearance of a protein matrix as well as its calcification (Pfeiffer and Gardner 1938 Clavert 1942)

(vii) *The Adrenals* These glands are always greater in females than in males The width of the adrenal cortex varies in the course of the ovarian cycle in the rat it is larger during oestrus either natural (Andersen and Kennedy 1932) or artificial (Bourne and Zuckerman 1940) An adrenal hypertrophy with a selective hyperplasia of the cortex results from any treatment with oestrogens (Allen and Bern 1942 Tuchmann Duplessis Aschkenasy Lelu and Aschkenasy 1948)

(viii) *The Hypophysis* This is hypertrophied in animals treated with oestrogens (Baker and Everett, 1944) There is an increase not only in the anterior lobe but also in the neuro-hypophysis (Stutinsky 1953)

On the whole the anabolic action of oestrogens is limited in mammals to a few tissues especially to genital organs and mammary gland

That is why in the guinea pig under the effect of oestrogens the incorporation of ^{14}C-labelled glycine increases in the proteins of the uterus and of the pubic symphysis but not in those of the xyphoid process, liver muscles or kidneys (Frieden, 1956)

(b) Organs and Tissues Unfavourably Influenced

According to Zondek (1936) in young rats treated with folliculin if one excepts the hypertrophied adrenals and hypophysis all internal organs are smaller and lighter than in the controls the kidneys and the pancreas being proportionally more affected than the others But the skeletal growth is the first to be inhibited by oestrogens.

(i) *Epiphyseal Cartilages* Zondek (1936) obtains dwarf rats after treat [illegible] to 40 g with foll [illegible] for 3 to 4 [illegible] harmonious [illegible] head [illegible] the thor

acic circumference is smaller the tail is shorter All the bones are reduced but have a higher calcium content which makes them more opaque to X rays This dwarfism is induced by a direct inhibiting action of oestrogens on the proliferative zone of the epiphyseal cartilages which becomes sclerosed while the bony matrix accumulates calcium

(ii) *The Testis* This organ atrophies under the action of oestrogens (Laqueur Hart and de Jongh 1926 Moore and Price 1932 Halpern and D Amour 1934) The involution of the seminal cells justifies the practice of hormonal castration of males with oestrogens

(iii) *The Thymus* Carrière Morel and Gineste (1937) note that folliculin induces a regression of the thymus in young rats male or female normal or castrated According to Schacher Browne and Selye (1937) the thymic involution provoked by oestrone is not mediated by the adrenals because it has also been observed in adrenalectomized rats Numerous authors have confirmed the involution of the thymus by oestrogens which are active even at very small doses (Kochakian 1947) Yet malignant hyperplasia of the lymphoid organs is on the contrary stimulated by oestrogens Thus Lacassagne (1937) observes the appearance of lymphoid sarcoma after a protracted treatment with oestrogens in mice belonging to strains where carcinoma does not occur normally the starting point of the neoplasm seems to have been most often located in the thymus

(iv) *The Salivary Glands* There is a sex difference in the weight and the enzyme content of the salivary glands in the rat and in the mouse (Lacassagne 1940 Raynaud and Rebeyrotte 1949) Their smaller dimension in the female may result partly from the lower alimentary consumption

(v) *The Hair* High doses of oestrogens inhibit the growth of hair in the rat (Zondek 1936 Emmens 1942 Hooker and Pfeiffer 1943) and in the dog (Gardner and De Vita 1940 Mulligan 1943) However in the human species it is chiefly the topographical distribution of the hair which is under the control of the sex hormones the hair of the head does not react to these hormones in the same manner as the hair of the body

(c) *Organs and Tissues with Variable or Disputed Receptivity*

(i) *The Liver* According to Kochakian (1947) the weight of the liver does not undergo much change in the male castrated mouse treated with oestrogens—its variations are parallel to those of the body The alkaline phosphatase activity is increased by low doses and the arginase activity is slightly increased by either low or high doses There appears to be no significant change in the protein content

On the other hand Griffiths Marks and Young (1941) report that fasting rats treated with oestrogens show an increase of the liver weight at the same time as of the glycogen stores

Fry Miller and Long (1942) observe a significant increase of the liver proteins in fasting male rats 30 hours after injections of stilboestrol (5 mg per 100 g)

In rats recovering from a protein deficiency and receiving low doses of oestradiol benzoate (12 rat units three times weekly) Aschkenasy and Dray (1954) find a significant increase in the liver weight as compared to the controls

Finally according to Glasser (1954) the liver is heavier in rats injected with stilboestrol (0 1 mg per day) than in pair fed controls and this difference still persists 30 days after the last injection Stilboestrol has an anticatabolic effect on the total proteins of the liver—it delays their loss induced by caloric restriction

Moreover it is to be pointed out that Gardner Allen and Smith (1941) have observed an hyperplasia and an hypertrophy of the biliary ducts in mice treated with oestrogens

(ii) *The Kidney* Feyel (1942) reports that oestradiol benzoate increases the weight of the kidneys in the female mouse more than testosterone propionate does in the male However according to Kochakian (1947 Kochakian *et al* 1948) none of the oestrogens tested (oestrone equilin oestriol α-oestradiol and its benzoic and dipropionic esters) influences significantly the kidneys of the male castrated mouse

On the contrary in rats recovering from a protein starvation Aschkenasy and Dray (1954) find the kidneys significantly increased under the effect of low doses of oestradiol benzoate

(iii) *The Spleen* This organ is increased in weight as compared to that of controls in rats treated with oestradiol (Aschkenasy and Dray 1954) but this effect is due only to an increase in the water content of the organ

(iv) *The Serum Proteins* According to Danforth *et al* (1946) the plasma proteins undergo in women variations related to the phases of the sexual cycle they decrease before menstruation at the same time as the body weight increases Friedberg and Greenberg (1947) find that oestrogens decrease the amino nitrogen content of the blood, in the rat But the electrophoretic pattern of the serum proteins is not significantly modified by these hormones in the same species (Hoch Ligeti and Irvine 1954)

(v) *The Thyroid* Every possible effect of oestrogens on the thyroid has been reported

Reiss and Pereny (1928) note that oestrogens inhibit thyroid activity According to Mercier Parot and Tuchmann Duplessis (1951 1952, 1953) a low dose of oestradiol benzoate induces a noticeable increase in

thyroid activity in the male castrated rat a high dose also stimulates the thyroid of the normal rat With MDDA low doses stimulate high doses inhibit the thyroid

During recovery from a protein deficiency rats receiving oestradiol benzoate have a heavier thyroid than their controls (Aschkenasy and Dray 1954) Hartsook and Magruder (1956) also find that the thyroid hypertrophies at the same time as the basal metabolic rate increases in rats treated with oestrogens

According to Eskin and Bogdanove (1956) the hyperplasic response of the rat s thyroid to propyl thiouracil is inhibited by oestradiol benzoate But after removing one of the two lobes of the gland in the rat, the compensatory hypertrophy of the remaining lobe may be stimulated by diethylstilboestrol (DES) provided that the animals are forcibly fed If they are allowed to decrease their intake *ad libitum* DES on the contrary inhibits the compensatory hypertrophy (Clifton and Meyer 1956)

On the whole the conflicting results mentioned above appear to arise both from the differences in the doses used and from the undernutrition which normally accompanies the treatments with oestrogens According to Noach (1955) the action of oestrogens on the thyroid involves—(i) the potentialization of the action of TSH on the thyroid and (ii) the inhibition of the release of this hormone

(vi) *The Haematopoietic Organs* By stimulating the ossification the oestrogens reduce the number of the haematopoietic cells of the bone marrow This is the reason why anaemia has been observed in various species treated with these hormones (cf Aschkenasy 1952) Thus Aschkenasy and Pariente (1953) increased the anaemia in castrated and thyroidectomized male rats subjected to a protein starvation by giving oestradiol (24 rats units every second day) for 4 weeks

However Jacobson (1944) does not record any significant anaemia in mice treated with oestradiol in spite of a marked ossification of the medullary canals

According to Feuchtinger (1940) the noxious effects of high doses of oestradiol in the dog and in the rat must be opposed to the stimulating action of low doses

Aschkenasy and Dray (1954) find that the oestrogens, even at low doses have an adverse effect on the regeneration of erythrocytes and neutrophils in rats recovering from a protein starvation.

(vii) *The Prostate and the Seminal Vesicles* These two male accessory sexual organs atrophy according to some authors (Korenchevsky and Dennison 1934 Zondek 1936) after treatment with oestrogens. On the other hand Kochakian (1947) finds that they tend to increase chiefly after low doses of these hormones. In fact it is the connective stroma and, to a lesser degree the muscular fibres of these organs which show hyperplasia (Freud 1933 Morrell and Hart, 1941) The effects depend to a large extent on the duration of the treatment—Lacassagne (1933) has observed in mice receiving oestrogens an atrophy of the prostate and of the seminal vesicles after 2 weeks, whereas 3 months later the dorsal prostate was on the contrary abnormally increased.

(viii) *The Muscles* According to Papanicolaou and Falk (1938) as well as Kochakian (1946) the skeletal muscles are not influenced by oestrogens Nevertheless, Pizzolato and Beard (1939) mention theelin (amniotin) among the sex hormones which increase the creatine in the muscle

2 Birds

(a) *Organs and Tissues Selectively Stimulated*

(i) *The Embryonic Gonadal Tissue* The strong morphogenetic action of oestrogens on the gonadal tissue of birds in tissue culture has been demonstrated by Wolff and Haffen (1952) and Stenger Haffen (1957) at the same time as the direct action of these hormones on the sexual differentiation of the embryo Also using the tissue culture method Scheibpflieger (1955) in a study of the differentiation of the Müller ian canal in the chick embryo observed that in opposition to testosterone oestradiol has a protective and stimulating effect on the growth of this tissue maintaining or increasing its nitrogen content

(ii) *The Oviduct* The growth of the oviduct is conditioned by oestrogens (Hertz and Sebrell 1944) which control also the production of the avidine of the egg white secreted by this organ.

(iii) *The Liver* This organ is hypertrophied in the chick receiving oestrogens (Breneman 1942) and this is not due only to the increase in its lipid content In pigeons receiving oestradiol benzoate (0 25 mg per day) Clavert (1944) observes that the liver may increase by 60 per cent as compared to controls this hypertrophy is expressed histologically by an increase in the number and the volume of the cells According to Common, Bolton and Rutledge (1948) there is at the same time in the chick a marked increase in the total proteins of this organ. This simultaneous rise of the ribonucleic desoxyribonucleic acid ratio noticed by Chapman *et al* (1949) clearly indicates that there is stimulation of cytoplasmic protein synthesis

(iv) *The Pancreas* This also is increased in weight in the chick treated with oestrogens (Breneman, 1942)

(v) *The Intestinal Tract* This is increased in weight and in length (Breneman, 1942)

(vi) *The Serum Proteins* Clavert and Duval (1944) and Mandel, Clavert and Mandel (1947) have shown that the plasma protein concentration is increased by

100 per cent at the beginning of an oestrogen treatment in pigeons (0 25 mg per day of oestradiol) this effect appears immediately but diminishes with a protracted treatment It concerns only the albumin fraction while the globulins remain unchanged Increase of albumin has been found also by several other authors

(vii) *The Adrenals* As in mammals the oestrogens induce an adrenal hypertrophy in birds (Breneman 1942)

(b) *Organs Unfavourably Influenced*

(i) *The Testis* Oestrogens inhibit the growth of the testis and suppress its function which explains the practice of the hormonal castration on the cockerel At the same time there is regression of some sex linked organs such as the comb (Emmens 1938 Breneman 1942)

(ii) *The Feathers* The oestrogens inhibit the formation of new pinfeathers but stimulate the growth of those already erupted (Lorenz, 1954)

(c) *Organs and Tissues with Variable or Disputed Receptivity*

(i) *The Thyroid* According to Emmens (1938 1939) the thyroid is sometimes increased in weight, but is always inhibited in its activity in the cockerel receiving oestrogens The gland seems to be also inhibited in male fowl treated orally with oestrogens as indicated by the decreased cardiac rate suggesting a decrease in the basal metabolic rate (Bird 1946) Epstein and Wolterink (1949) find a decrease in the rate of thyroidal ^{131}I uptake in the chick

(ii) *The Haematopoietic Organs* The bone marrow is often inhibited by the intense osteogenesis due to the oestrogens which would explain the anaemia reported in the domestic fowl by Taber Davis and Domm (1942) However Benoit Messerschmidt and Grangaud (1941) find on the contrary a stimulation of the erythro- and myelopoiesis in the bone marrow of ducks receiving repeated injections of oestradiol.

E. Factors Influencing the Metabolic Action of the Oestrogens

1 Dietary Factors

(a) *Total Intake of the Ration*

In animals in which the oestrogens have a depressive action on the appetite any treatment with these hormones induces undernutrition. We have seen that in certain experiments the metabolic effects obtained may be reversed by forcibly feeding the animals—action on the growth (Ingle 1941 Ingle *et al* 1947) action on the compensatory hypertrophy of the remaining thyroid lobe after removal of the other (Clifton and Meyer 1956)

(b) *Proteins in the Diet*

The sensitivity of the vagina to oestrogens is not suppressed by a protein starvation of 44 days (Arvy, Aschkenasy Aschkenasy Lelu and Gabe 1946) whereas the oestrus cycle disappears already after 20 to 40 days of such a diet (Aschkenasy 1946)

The weight loss provoked by stilboestrol is larger in protein-deficient rats than in normally fed controls (Glasser 1957) A protein starvation inhibits also the anticatabolic action of stilboestrol on the proteins of the liver (Glasser 1954 1957)

The adrenal hypertrophy induced by stilboestrol is recorded even in protein deficient rats but the weight increase is less than with a normal diet (Glasser and Leathem 1955), the stimulation by oestrogens of the weight gain in cattle appears only in the presence of a sufficient protein intake (Struempler and Burroughs 1956 Reynolds *et al* 1956)

(c) *Vitamins*

(i) *Folic Acid* Hertz and Sebrell (1944) and Hertz (1945) demonstrated the interference of folic acid with the sensitivity of tissues to oestrogens The hypertrophy of the genital tract of the hen under the action of DES does not occur if the diet is deficient in folic acid either directly or by administration of an anti-folic agent (Hertz, 1948 Hertz and Tullner 1949) The response of the same tissue to a natural oestrogen (oestrone) also needs the presence of folic acid (Kline and Dorfman 1948 1951)

The same intervention of folic acid has been found in mammals—monkey (Hertz, 1948 Zarrow Hisaw and Salhanick 1951) and rat (Hertz and Tullner 1949 Yadu and Meites 1949) and also in other vertebrates such as the frog (Goldsmith Schreiber and Nigrelli 1948)

(ii) *Vitamin B_{12}* According to Kline (1955) vitamin B_{12} also interferes in the growth response of the oviduct of the hen to stilboestrol Meites (1952) found that injections of this vitamin partially compensate the inhibiting action of stilboestrol on the appetite and the growth of the young male rat but this result was not confirmed by Glasser (1954 1957)

2 Hormonal Factors

Many indirect hormonal actions have been held responsible for the effects exerted by oestrogens on the growth of the whole body or of different tissues—action on the hypophyseal growth hormone on the thyrotropic hormone and the thyroid and especially on the adrenals through a discharge of ACTH

Thus Gaarenstrome and Levie (1939) as well as Richards and Kueter (1941) affirm that the hypophyseal growth hormone may compensate the inhibition of the growth of young rats receiving high doses of oestrone

But it is especially the increase in the adrenal corticoids following a discharge of ACTH which might explain certain effects of oestrogens Fry Miller and Long (1942) use the term corticomimetic to designate the action of the oestrogens on the nitrogen and carbohydrate metabolism Thirty hours after injection of a high dose of stilboestrol (5 mg per 100 g) male fasting rats show a weight loss and an increase of urinary nitrogen and potassium excretion of the glycogen and protein contents of the liver and also of the blood glucose and serum potassium Now, the same dose of stilboestrol does not induce these changes in adrenalectomized animals Likewise after hypophysectomy oestrogen injections increase neither the liver glycogen nor the nitrogen excretion

The authors suggest that the action of stilboestrol is exerted through the hypophyso-adrenocortical system This has been confirmed by Gemzell (1948) for the increase in the liver glycogen under the action of the oestrogens

Some other actions may be explained in the same way

The stimulating effect of the oestrogens on the mitotic index of the epidermis is followed by a depressive effect the latter appears from the moment when the adrenal cortex has grown larger furthermore adrenalectomy prevents this secondary depression (Bullough 1952) The inhibiting effect of oestrogens on the hair growth could be explained in the same way (Baker and Whitaker 1949 Ingle and Baker 1951)

It is probably for the same reason that the appetite at first strongly inhibited in the rat treated with oestrogens is partially reactivated if the treatment is protracted and thus the protein intake increases progressively (Aschkenasy Lelu 1951) On the other hand the involution of the thymus under the action of oestrone does not occur through the adrenals because it is recorded even in adrenalectomized animals (Schacher Browne and Selye 1937)

In addition the problem of the interactions between oestrogens and adrenals is related to the question whether these hormones increase or on the contrary diminish the resistance to stress even though they are themselves already a stressing factor According to Patt *et al* (1949) oestrogens increase the survival time of mice exposed to X rays but Campbell Bern and De Ome (1956) find that they lower the resistance of animals to cold or formalin injections

F Conclusion on the Metabolic Action of the Oestrogens

In mammals, the oestrogens exhibit at the same time—

(i) An anabolic action on the growth of certain specialized tissues (female effectors and connective stroma of some male accessory organs)

(ii) With *high doses* an inhibition of the total development of the body with an increase in the urinary nitrogen excretion

(iii) With *low doses* a slight anabolic effect on the body as a whole, with an improvement of the nitrogen balance this effect being still a subject of controversy

These contradictory properties may be connected—

(*a*) for the action of high doses with a sharp and immediate reduction of the dietary intake leading to undernutrition or even with a toxic action of such hormones

(*b*) with stimulations or inhibitions of some enzymatic mechanisms

The extremely rapid inhibiting action on the appetite could result from the stimulation by the oestrogens of the hypothalamic nuclei, which seem to control the mechanism of satiety

We have already pointed out the oestrogen induced changes in the amounts of some enzymes in different organs but it is difficult to connect these changes with the synthesis of new tissues

On the other hand the oestrogens being dissimilar as to their chemical structure there is little chance of finding modifications related to one specific enzyme However Fishman and Fishman (1944) find that β glycuronidase shows a true specificity towards the oestrogens both natural and synthetic. After ovariectomy in the rat it decreases in the uterus and in the vagina and it may be increased again by oestrogens. However the enzyme is not influenced in other tissues —liver kidney spleen or blood (Fishman 1947) According to this author (1951) the response of β glycuronidase to oestrogens is not related to variations of nitrogen and has probably nothing to do with the growth of the tissue

Most of the direct actions exerted by oestrogens *in vitro* are characterized by an *inhibition*—

(i) Wolff and Wolff (1952) show that the morphogenetic and differentiating action of oestradiol benzoate on an embryonic organ of a bird (the syrinx of the duck) in tissue culture is an inhibiting one Oestradiol acts by inhibiting the normal differentiation of the organ which in a standard medium, would occur following the male type the female form appears as the result of an inhibition

The use of a relatively poor medium, containing only cysteine as the sole nitrogen donor gives the same inhibition as the addition of female hormone (Wolff 1957)

(ii) Oestradiol possesses an inhibiting action at very low doses on the cellular division of the sea urchin embryo it acts both on the cytoplasmic structure and on the metabolism of DNA (Agrell, 1955)

(iii) Oestradiol inhibits the mitotic activity of a culture of rabbit fibrocytes (Debrunner 1953)

(iv) High doses of synthetic, but especially of

natural oestrogens significantly inhibit *in vitro* the anaerobic glycolysis of slices from a human prostatic adenoma low concentrations in contrast sometimes stimulate and increase this same glycolysis (McDonald and Latta 1956)

It is not impossible that all these effects may be explained in terms of the same general phenomenon—

Oestrogens are *in vitro* antioxidizing agents The succinic oxidase and malic oxidase systems are inhibited *in vitro* by the oestrogens (more strongly by the synthetic ones) either directly or by interaction with the cytochrome oxidase of these systems (McShan and Meyer 1946 Meyer and McShan 1950)

IV GENERAL CONCLUSION

The action of the sex hormones on the nitrogen metabolism is not represented by a general anabolic potency towards the whole body

The androgens reduce the nitrogen catabolism but this action is only transitory The oestrogens perhaps slightly anabolic at low doses are always catabolic at high doses

In fact these variable effects on the total nitrogen balance result from intricate stimulations and inhibitions exerted by these hormones on the development of various tissues

Androgens selectively increase the growth of the male accessory genital tissue the kidneys the haematopoietic marrow the salivary glands and also some muscles But on the other hand they have an unfavourable effect on the lymphoid organs chiefly the thymus

On their part oestrogens enhance the growth of the female genital tract and perhaps that of the liver and stimulate also the anabolism of plasma proteins in birds On the other hand oestrogens have a noxious effect on the skeletal growth on the bone marrow thymus testis and salivary glands

Under the action of the sex hormones an internal reshuffling occurs between the proteins of different tissues Probably it is through disturbances in certain enzymatic systems that these hormones enhance or inhibit the tissue growth But up till now no specific action on a given enzyme could be connected with the tissular effects of these hormones

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The Biosynthesis of Vitamin C (Ascorbic Acid)

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Contents

I INTRODUCTION

The records of history and observations on the food practices of primitive man in his efforts to prevent or cure scurvy illustrate the unique association of vitamin C with fresh foods of either plant or animal origin Recognition of its almost universal occurrence in all living organisms was not established however until the vitamin had been identified and precise chemical methods of measuring its content in natural products *had been used extensively* (Lind *Bicentenary Symposium*, 1953 McCollum 1958, King 1936 1939)

Among the early demonstrations of biosynthesis were observations that sprouting seeds accumulated the vitamin rapidly from inactive stored material (Hess 1920) and that although rats thrived indefinitely on a diet apparently free from the vitamin their tissues fed in small quantities to guinea pigs protected *them from scurvy*

Staining methods for observing the distribution and relative concentrations of the vitamin in tissue slices were developed extensively by Bourne (1936) Giroud and Leblond (1937) and their associates based on the use of acid solutions of silver nitrate

Reliable and sensitive chemical measurements of the vitamin in biological materials have been developed chiefly by *adaptations of two general procedures* Both types of reagents have been adapted to macro and micro-scale estimations for use with a great variety of materials The first method to be widely adopted for direct titration was introduced by Tillmans Hirsch and Hirsch (1932) based on the reduction of the dye 2 6-dichlorophenolindophenol by the vitamin Distinct improvements in the method which made it *generally applicable were then made* by Harris and Ray (1933) Bessey and King (1933) Glick (1949) and subsequently by others A second procedure was introduced by Roe and Kuether (1943) based upon conversion of ascorbic acid to dehydroascorbic acid and condensation with 2 4-dinitrophenylhydrazine followed by addition of sulphuric *acid to develop a colour reaction* (Lowry, Lopez and Bessey 1945)

The above methods of analysis when used with adequate precautions have furnished an extensive body of reliable information on the occurrence synthesis and metabolism of ascorbic acid and dehydroascorbic acid in plants and animals but unfortunately the apparent simplicity of these and other *methods has led many authors to publish data that* cannot be relied upon The most frequent errors and the greatest in magnitude have resulted from the interference of sulfhydryl and dienolic groups in the dye titration of ascorbic acid In addition to the R-SH compounds natively present in many tissues treatment of extracts with hydrogen sulphide to reduce dehydroascorbic to ascorbic acid before titration *often is accompanied by partial conversion of carbonyl* groups to R-SH groups This reaction is characteristic of many aliphatic ketones and aldehydes and quinones Errors introduced by the reducing action of dienols are less frequent in studies of natural products but readily arise when reducing sugars are fermented or heated in alkaline media

The evidence based chiefly on careful analytical and histochemical studies indicates however that ascorbic acid is a characteristic cellular constituent of all the higher animal and plant forms thus far investigated The range of study has included many types of bacteria yeasts moulds algae lichens diatoms protozoa earthworms insects marine invertebrates sea urchins molluscs crustaceans crabs fishes birds and mammals In many instances this kind of *evidence should be re studied with the new and much* more critical techniques using isotope labelled precursors

The only types found thus far to be without a normal capacity to synthesize the vitamin from common precursor nutrients are man and other primates and guinea pigs

II EARLY TESTS BASED ON TITRATION MEASUREMENTS AND KNOWN SUBSTRATES

One of the earliest convincing tests of a specific precursor for ascorbic acid synthesis was reported by Reid (1938) and by Reid and Robbins (1942) using a simple sugar (glucose or sucrose) as a carbon source in a nutrient medium for the growth of root tips Growth was accompanied by rapid and extensive synthesis of the vitamin

Although many reports claimed a special role for mannose in the synthesis other studies failed to confirm the reports (Mapson Cruikshank and Chen 1949 Hawthorne and Harrison, 1937 Scheunert and Schieblich 1937) The early tests with sugar substrates were often disappointing because it seemed impossible to accelerate the synthesis sufficiently to give satisfactory evidence In general, synthesis tended to be characteristic of growth rate or metabolic activity (especially in glandular tissues) and only slightly responsive to added test precursors (King, 1936 1939) There was some indication of an association with anaerobic glycolysis rates (Williams 1943)

When urinary excretion rates in relation to dietary intake were studied in albino rats striking differences were observed but there was no immediate clear evidence to indicate whether the effects were dependent upon differences in precursor intake or whether they might have a different origin Such diverse materials as rolled oats oat oil distillate from halibut liver oils essential oils dried grass or green leaf oils caused an increased ascorbic acid excretion about equal to feeding 25 mg of the vitamin per day when added to a whole milk diet (Musulin *et al* 1939) Guinea pig feeding tests demonstrated (by assay) close agreement with the titration values

Identification of some of the pure compounds e g hexenal which were responsible for the greatly increased excretion (10 to 100 times on feeding 50 mg/day) then led to a systematic study of relation ships between structure and enhanced excretion (Musulin *et al* 1939) Ketones such as carvone piperitone β ionone isophorone and camphor were especially active and simpler aliphatic compounds such as dipropyl ketone and dimethylacetyl-carbinol were distinctly active Because of the wide variation in structure and the striking effects exerted by substances with carbon chains less than six carbons in length it did not seem likely that the substances were serving as precursors The next series of compounds tested included barbiturates chloretone and a number of representative nerve depressants nearly all of which exerted a marked effect upon ascorbic acid excretion Other laboratories observed comparable effects with analogous or identical products and added support for four lines of evidence —(*a*) the most active compounds were not serving directly as precursors (*b*) the biochemical changes induced bore a close relationship to suppression of nerve function (*c*) there was a close parallelism between increased ascorbic acid excretion and glucuronic acid excretion and (*d*) since the increased excretion far exceeded the initial body content and could be maintained through long periods with an accompanying moderately increased tissue concentration it seemed likely that the excretion was a true reflection of increased synthesis Recent measurements of the body pool and turnover rate of ascorbic acid in rats also indicate that the increased excretion of ascorbic acid after chloretone or pentobarbital administration is the result of accelerated biosynthesis (Burns, Mosbach and Schulenberg 1954)

In vitro studies of ascorbic acid synthesis from representative sugars and sugar fragments showed only moderate increments for ascorbic acid synthesis by tissue slices and emulsions Tissues excised from animals pretreated with chloretone or phenobarbital showed a moderate increment in the rate of ascorbic acid synthesis compared with tissues from normal animals Inanition of the animals sufficient to lower the glycogen reserves induced sharply decreased synthesis in the tissues but none of the specific substrates added resulted in increments in ascorbic acid yields that were markedly in excess of the yields furnished by glucose or by a combination of glucogenic fragments such as dihydroxyacetone glyceraldehyde or mixtures of these compounds with pyruvate In liver brain, or kidney tissues from chloretone treated albino rats, the addition of glucose or glucose fragments resulted in fairly consistent but moderate increments in the *in vitro* formation of ascorbic acid in contrast with guinea pig tissues which did not show a comparable response (Smythe and King 1942)

Parallel studies by Lipschitz and Bueding (1939) and Mawson (1935) gave supporting evidence for the parallelism with glucuronic acid formation, and for glucose precursor relationships to ascorbic acid.

III INVESTIGATIONS BASED ON KNOWN SUBSTRATES AND ISOLATED PRODUCTS

At this stage in the investigations radiocarbon became available as a means of tracing known compounds with ^{14}C *in specific positions* so that intermediate and end products might be isolated and the position of ^{14}C established by degradative procedures thus furnishing definitive information concerning relation ships between precursors and end products.

Uniformly labelled [^{14}C]glucose administered to guinea pigs previously treated with borneol permitted *isolation of bornyl glucuronide* in 24-hour urine specimens containing approximately 1 8 to 4 0 per cent of the administered [^{14}C]glucose The distribution of ^{14}C in position 6 compared with the remainder of the glucose molecule indicated that there had been no change in the ^{14}C distribution in the carbon chain. Administration of [1 ^{14}C]L ascorbic acid did not result in transference of detectable quantities of ^{14}C into the bornyl glucuronide during the subsequent period thus indicating that the vitamin did not serve as a precursor of glucuronides (Mosbach and King, 1950) This result stood in sharp contrast to the subsequently observed *conversion of* both glucose and glucuronic acid lactone to ascorbic acid

Investigations of glucose and glucuronic acid lactone conversion to ascorbic acid and glucuronides then revealed that distinct strain differences exist in comparing Wistar strain albino rats with those generally referred to as the Sherman strain. The responses to chloretone and borneol developed further interesting relationships to both of the acid end products. When maintained on an evaporated milk

diet and fed 45 mg of chloretone daily the Sherman strain rats excreted much less ascorbic acid and more glucuronic acid than was observed for Wistar strain animals under identical conditions Borneol exerted only a moderate effect upon ascorbic acid excretion in either strain of animals but resulted in a greatly enhanced excretion of glucuronic acid in both strains After chloretone administration for example the Sherman strain animals showed respectively increments in ascorbic acid and glucuronic acid approximately 5 times and 2 times the quantities excreted without the supplement In contrast the comparable figures for the Wistar strain animals were 50-fold and 12 fold (Mosbach Jackel and King 1950)

To make a critical test of the possibility that ^{14}C from labelled chloretone might give an indication of direct transference of a fragment to ascorbic acid methyl labelled [^{14}C]chloretone was prepared and administered in the usual way followed by isolation of the resultant ascorbic acid There was no detectable transference of ^{14}C within a period of 24 hours

Uniformly labelled [^{14}C]glucose prepared by photosynthesis showed a conversion of 0 3 per cent to ascorbic acid isolated from the urine within the same period This ratio of ^{14}C transference from glucose to ascorbic acid was approximately equivalent to the total calculated conversion from the quantity of carbohydrate consumed during the same period Partial degradation of the biosynthetic ascorbic acid showed clearly that the ^{14}C content in positions 1 and 2 had remained identical with the activity in the initial glucose (Jackel *et al* 1950)

Administration of 2 0 to 50 0 mg of total ascorbic acid together with appropriate quantities of [1 ^{14}C]L-ascorbic acid followed by measurements of respiratory activity excretion levels and studies of the distribution of the labelled vitamin in many of the tissues resulted in clear evidence that the excess quantities of ascorbic acid were converted largely to $^{14}CO_2$ within 24 hours The quantities of stored [^{14}C]ascorbic acid in body tissues or excreted followed a normal path for the unlabelled ascorbic acid as measured by titration There was no indication of major transference of ^{14}C to compounds other than ascorbic acid or into any of the tissues except in the case of oxalate excretion which accounted for approximately 1 per cent of the total ^{14}C administered and approximately 30 to 40 per cent of the ^{14}C contained in the urine It was of special interest to note that there was no tendency for transference of ^{14}C into collagen or chondroitin sulphate and only very small quantities remained in the liver glycogen Nearly all of the ^{14}C contained in the soft tissues could be readily extracted with water or 8 per cent acetic acid solution and recovered as ascorbic acid by carrier dilution (Burns Burch and King 1951)

To permit more rigid appraisal of the apparent direct transference of the 6-carbon glucose chain to ascorbic acid in the albino rat by a series of postulated reactions in which carbon 1 in D-glucose would become carbon 6 in L ascorbic acid [1-^{14}C]glucose was prepared and administered to rats in parallel with chloretone treatment to furnish relatively high yields of the vitamin Administration of the glucose and subsequent isolation of the excreted ascorbic acid followed by degradation and isolation of carbons 1 2, and 6 gave confirmatory evidence of a direct conversion without carbon-chain cleavage

Subsequent tests with [6-^{14}C]glucose followed by isolation and degradation of the resultant [1 ^{14}C]-ascorbic acid gave further evidence in support of a direct conversion of glucose to ascorbic acid without major changes in the position or configuration of the glucose molecule other than those necessary for the respective oxidation and reduction changes The relative yields were very similar to those obtained in the earlier experiments The similarity in structures of D glucuronic acid lactone and ascorbic acid together with the evidence of their metabolic relationship to D-glucose and excretion stimulants strongly suggested the possibility that D-glucuronic acid or its lactone might be an intermediate on the pathway to ascorbic acid Tests with uniformly labelled [^{14}C]D-glucuronic acid lactone (glucurone) gave supporting evidence for the relationship in that the conversion was 4 to 8 times as large for glucurone as had been found for glucose and degradation studies gave similar evidence of there having been no change in the arrangement of carbons within the molecule (Horowitz, Doerschuk and King, 1952 Horowitz and King 1953*a* 1953*b*)

Burns and Evans (1956) found that carboxyl-labelled D-glucurono-lactone yielded carboxyl labelled L ascorbic acid in both normal and chloretone treated rats

The consistent yield of small quantities of [^{14}C]-oxalate from ascorbic acid led to further studies of preformed oxalate and [1 ^{14}C]dehydroascorbic acid Investigations of [^{14}C]oxalic acid indicated that there was practically no conversion to respiratory $^{14}CO_2$ although small amounts of deposited oxalate were distributed widely in rat tissues with the highest concentration in bone tissue Measurements of the half life of [^{14}C]oxalic acid indicated a value of approximately 2 5 days compared with 3 6 days for the half life of ascorbic acid and a turnover rate of approximately 5–8 mg of ascorbic acid per day Separate tests showed no significant conversion of [^{14}C]2 3-diketogulonic acid to ascorbic acid in the rat (Curtin and King, 1955 Weinhouse and Friedmann, 1951 Vickery and Abrahams 1950 Burns Mosbach and Schulenberg, 1954 Boxer and Stetten, 1944)

Parallel experiments to furnish an insight into the metabolic relationships of glucuronic acid as an intermediate between glucose and ascorbic acid showed equally clear evidence for the conversion of [1-^{14}C]-glucose to [1-^{14}C]glucuronic acid (60 per cent yield of total ^{14}C in position 1) and [6-^{14}C]D-glucose to [6-^{14}C]D-glucuronic acid (67 per cent yield of total activity in carbon 6). However, when uniformly labelled [^{14}C]D-glucurone was administered to borneol fed guinea pigs, the subsequent degradation studies indicated that the ^{14}C transference to positions 4, 5 and 6 was distinctly less (average 12 per cent each) than for positions 1, 2 and 3 (average 21 per cent each). Partial degradation of glucose from the stored glycogen subsequent to feeding the labelled glucurone showed approximately the same ^{14}C value in position 6 as had been found in the excreted glucuronide. When [6-^{14}C]glucurone was administered subcutaneously to borneol fed guinea pigs, more than 26 per cent of the ^{14}C was exhaled during the first hour and more than 50 per cent within 24 hours. One-third to one half was excreted in the urine within 24 hours (Douglas and King, 1952, 1953*a*, 1953*b*).

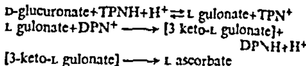
Packham and Butler (1952) observed a greater conversion of glucuronic acid to glucuronide products in naphthol stimulated rats.

Extensive observations on the presence in rat liver of an enzyme that catalyses the conversion of glucurone-γ-lactone to glucuronate, together with observations on the metabolism of glucurone, have been reported by Eisenberg and Field (1956) and other investigators (Butler and Packham, 1955; Rabinowitz, 1955; Brodie and Lipmann, 1955).

ul Hassan and Lehninger (1956) observed that rat liver extracts could convert D-glucuronic acid and D-galacturonic acid or their lactones to ascorbic acid but did not find evidence to support the concept that uridinediphosphoglucuronic acid participated in ascorbic acid formation. After dialysis of the extracts it was necessary to add adenosine triphosphate, diphosphopyridine nucleotide, Mg^{++} and nicotinamide to restore full activity. Both L-gulonic and L-galactonic acid and their respective lactones were converted to ascorbic acid at rates suggesting their activity as intermediates between the uronic acids and ascorbic acid. This mechanism had been postulated previously by Isherwood, Chen and Mapson (1954*a*).

Conversion of D-glucuronolactone to ascorbic acid was similarly demonstrated to occur in liver extracts of mouse, dog and rabbit, but not from guinea pig liver (ul Hassan and Lehninger, 1956). Chatterjee *et al.* (1957) reported an increased yield of L-ascorbic acid from D-glucuronolactone in the presence of very small concentrations of cyanide.

Lehninger and his associates (Grollman and Lehninger, 1957; Bublitz, Grollman and Lehninger, 1957) have extended the enzymatic investigations of ascorbic acid synthesis to include suggestions of the following types of reaction—



D-glucuronate + TPNH + H^+ ⇌ L-gulonate + TPN^+

L-gulonate + DPN^+ ⟶ [3-keto-L-gulonate] + DPNH + H^+

[3-keto-L-gulonate] ⟶ L-ascorbate

Decarboxylation of 3-keto-L-gulonate was postulated as a possible means of forming L-xylulose and carbon dioxide as an alternative reaction course in addition to L-ascorbic formation.

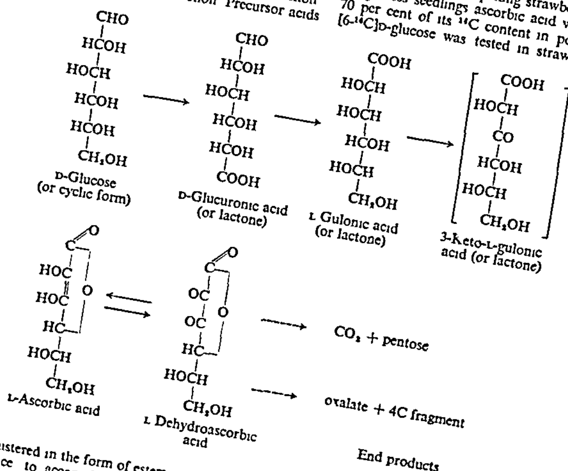
Animals capable of synthesizing ascorbic acid under physiological control, such as the rat, contain at least the respective enzyme systems indicated in liver cells, but may contain only two of the enzyme systems in kidney tissue. In contrast, they reported the presence of all three enzymes in the kidney tissue of chicks, pigeons and turtles. They postulated that in man and other primates and in guinea pigs, enzymes are present to carry forward only the first two reactions. Burns and co-workers reached similar conclusions based on ^{14}C studies both *in vivo* (Burns and Evans, 1956) and *in vitro* (Burns, 1957*a*, *b*; Burns, Peyser and Moltz, 1956) which demonstrated the conversion of L-gulonolactone to ascorbic acid in the intact rat and in rat liver homogenates, and essentially no conversion in the guinea pig, and in guinea pig, monkey and human liver homogenates.

Independent studies by Burns and co-workers using labelled intermediates have demonstrated the conversion of [1-^{14}C]D-glucose and [6-^{14}C]D-glucuronolactone to labelled L-gulonic acid in both rats and guinea pigs (Burns, 1957). Extracts from the livers of rats, monkeys and guinea pigs also accomplished the conversion of [6-^{14}C]D-glucuronolactone to L-gulonic acid (Burns, 1957). Direct conversion of the intact carbon chain of [1-^{14}C]L-gulonolactone to L-ascorbic acid was demonstrated by isolation of [1-^{14}C]L-ascorbic acid from normal and chloretone-treated rats (Burns and Evans, 1956). In extending these studies, Burns *et al.* (1956*a*) have also studied the enzyme system in rat liver which catalyses the conversion of [1-^{14}C]L-gulonolactone or the corresponding acid to labelled ascorbic acid. No conversion was observed in homogenates of kidney, brain, small intestine, and muscle.

Ganguli, Roy and Guha (1956) have given additional evidence concerning the role of adenosine triphosphate in the synthesis of ascorbic acid by chloretone-stimulated rat tissues. They also present possible explanations of the chemical reactions that are influenced by narcotic type reagents in their bearing on the glycolytic cycle.

Isherwood, Mapson and collaborators (1953*a*

1953b 1954a 1954b 1956 1957) observed in germinating cress seedlings a rapid conversion of D galactose and D glucose to ascorbic acid as measured by titration of the extracts and extended their investigations to include studies of the controlling enzymes synthesis by separated pea mitochondria and the testing of several postulated intermediates Comparative studies were also based upon synthesis by rat liver mitochondria and increased urinary excretion Precursor acids



were administered in the form of esters or lactones in each instance to accomplish cell penetration. The γ-lactones of D-glucuronic and L-gulonic acids D-galacturonic methyl ester and L galactono-γ-lactone were reported to be converted to ascorbic acid in cress seedlings and in rats An extensive series of other D- and L sugar acid lactones did not give rise to the vitamin

Summation of the evidence based chiefly upon studies in rats (and guinea pigs for contrast) and supported by studies in certain plants indicates that a major pathway can be outlined as shown below

However, Loewus and collaborators (Loewus Jang and Seegmiller, 1956 Loewus and Jang 1957 Loewus and Jang 1958) reported that from [1-^{14}C]D-glucose in detached ripening strawberries or germinating cress seedlings ascorbic acid was formed with 70 per cent of its ^{14}C content in position 1 When [6-^{14}C]D-glucose was tested in strawberries 73 per cent of the ^{14}C content of the ascorbic acid was in position 6 and 24 per cent was in position 1 Cleavage of the carbon chain was not indicated Comparable tests with [6-^{14}C]L idose did not result in significant quantities of [^{14}C]ascorbic acid From their observations of ^{14}C labelled patterns in ascorbic acid glucose xylose arabinose and galacturonic acid from labelled pentoses and hexoses, the authors suggest that the probable pathway is via 6-phosphogluconic acid or its lactone and glucose-6-phosphate

IV PRODUCTS OF ASCORBIC ACID METABOLISM

The carbon chain of [^{14}C]L ascorbic acid has been found to be converted almost entirely to carbon dioxide in the rat and guinea pig (Burns, Burch and King, 1951 Burns Mosbach and Schulenberg, 1954 Curtin and King, 1955 Rudoff Becker and King, 1956 Burns, Dayton and Schulenberg, 1956) but a portion of the carbon in positions 1 and 2 gives rise to oxalic acid. The resultant [^{14}C]oxalic acid is not an

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4

Treatment and Prevention of Kwashiorkor

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Contents

I INTRODUCTION

Knowledge of the biochemical physiological and pathological characteristics of kwashiorkor has grown rapidly since the appearance of the monograph on *Kwashiorkor in Africa* by Brock and Autret (1952) and has been summarized in a number of recent reviews (Trowell Davies and Dean 1954 Brock 1954 Scrimshaw *et al* 1956 Béhar *et al* 1956) Intensive study of the syndrome in many different parts of the world has resulted in a clarification of the features of kwashiorkor common to all regions in which it occurs as well as the identification of those associated signs and symptoms which are the result of local conditions This has been facilitated by the inter change of ideas at several international conferences and by the exchange of visits among workers from different countries There has also grown up a com mon body of successful experience in the treatment of kwashiorkor and a genuinely international approach to the problems of its prevention These developments have been stimulated and assisted to a precedent setting degree by two of the specialized agencies of the United Nations the World Health Organization (W H O) and the Food and Agricultural Organization (F A O) and both U N I C E F and U N E S C O are now co-operating in this effort

The specific purpose of this review is to describe present knowledge and concepts of the treatment and prevention of kwashiorkor derived from world wide investigations and experience For a systematic cover age of the clinical biochemical and pathological characteristics of the syndrome the reader is referred to the reviews listed reference to these aspects of kwashiorkor is made only as they relate to or serve to guide therapy or prevention

The authors have attempted not only to include controversial points and divergent opinions but also to evaluate them in the light of the latest knowledge available and the consensus of experienced workers In so active a field of research this desirable but difficult procedure will inevitably lead to some errors of both omission and commission It is hoped never theless that this type of summary at a time when international interest in the problem has been so greatly aroused, will contribute to the widespread efforts being made to reduce morbidity and mortality from kwashiorkor by disseminating knowledge of effective treatment and by instituting practical measures for prevention

II. CONCEPT OF KWASHIORKOR AS A DISEASE ENTITY

Kwashiorkor is recognized as a disease syndrome arising as result of severe deficiency of dietary protein relative to caloric intake and characterized typically by retardation of growth and development oedema apathy anorexia alterations in the colour and texture of the hair lesions of the skin and diarrhoea It occurs most commonly in young children because at this period of life protein requirements are relatively high and diets are most likely to be deficient in pro tein. It is frequently accompanied by deficiencies in other nutriments which differ somewhat from one region to another and complicate the clinical picture Not only do the associated deficiencies of vitamins and minerals vary but also the relative adequacy of the caloric intake When protein deficiency occurs in a child whose caloric intake is adequate the result is the classical type of kwashiorkor in which tissue wasting is not a conspicuous feature With excessive caloric intakes subcutaneous fat may be increased and the child develop the so-called sugar baby type of kwashiorkor (Fig 14 1) described in Jamaica (Jelliffe Bras and Stuart 1954) As kwashiorkor occurs throughout Latin America most of Africa India and the Middle and Far East it is combined with a defi ciency of calories sufficient to cause pronounced wasting and presents a continuous clinical spectrum merging with marasmus (Fig 4 2) These intermediate clinical forms representing combinations of marasmus and classical kwashiorkor could more properly be designated as marasmic kwashiorkor but since they are the prevalent form of the disease this is not customary Because it is the common type instruc tions for the therapy of kwashiorkor are generally given with the marasmic case in mind Fig 4 3 shows the underlying wasting evident in a child with maras mic kwashiorkor once the oedema is lost These concepts must be kept in mind since severe caloric deficiency complicates both treatment and prevention.

Regardless of the degree of associated caloric deficiency the fundamental aetiological factor in the development of kwashiorkor is a marked deficiency in the amino acids necessary for protein metabolism. This may come about because dietary protein is inadequate in quantity or quality but more frequently it is deficient in both total amount and biological value Any factor decreasing either nitrogen absorption or nitrogen retention will contribute to the development of protein deficiency and may precipitate kwashiorkor

in cases of border line protein intakes The high frequency with which diarrhoea of infectious origin and other infectious diseases decrease nitrogen retention prior to the appearance of kwashiorkor will be discussed in detail in the section on prevention It has not been possible to isolate the effects of single amino acids from those of the essential amino acids as a group Since unlike the vitamins a deficiency of one essential amino acid blocks the utilization of the others it is almost impossible to design suitable experiments on this point without jeopardizing the child

While some of the associated nutrient deficiencies depend on inadequacies in the local diets which are not necessarily constant from region to region others, like the deficiency of the fat soluble factors, vitamin A and E, appear to depend upon interference with intestinal absorption (Trowell Moore and Sharman, 1954 Arroyave *et al* 1959) Some deficiencies, such as those of iron and ascorbic acid may not be apparent at the time of the acute episode but develop rapidly if the therapeutic diet does not supply these factors in adequate amounts

III PRINCIPLES OF TREATMENT

A Dietary Measures

1 Meeting Protein Needs

(a) *The Amount of Protein*

All investigators are in agreement that large amounts of protein of high biological value are of primary importance in therapy but the actual amounts administered vary widely Carvalho (1947) reports satisfactory results with 3 to 4 g of milk protein per kg body weight per day and DeMaeyer (1954) recommends 4 5 g per kg per day Some investigators however on the basis of balance studies which indicate a direct relationship between the absorption and retention of nitrogen and the quantity of protein ingested advocate much higher protein dosages While Dean (1953) has used as much as 15 to 20 g per kg per day he admits that no further beneficial effect is obtained from such high protein intakes and recommends the use of 8 to 10 g per kg per day which is approximately the same dosage as is used by workers in India (Gopalan and Ramalingaswami 1955)

When the child consumes the food readily and milk is the major protein source we generally employ intakes varying between 5 and 7 g per kg per day Such levels are not usually attained however until the second week of treatment Owing to the profound anorexia intolerance to food and the danger of abnormal distention of the stomach and intestine it is necessary to initiate treatment at much lower levels of intake particularly in severe cases During the first 24 hours 1 to 2 g per kg can generally be administered and this amount increased from 2 to 5 g per kg during the second to fourth day of treatment It should also be clear that if intakes as high as 5 to 7 g of protein per kg are employed these will gradually drop as the child gains in weight and as nitrogen stores are filled Furthermore the successful treatment of kwashiorkor with mixtures of vegetable origin, even though the intake conveniently attainable does not exceed 3 to 5 g of protein per kg because the bulk of food which the child can consume is limited indicates that higher protein intakes are not necessary for complete and satisfactory recovery

Recommendations regarding protein intakes which are based on weight have the limitation that cases of kwashiorkor vary from normal or even excessive weight for age to extreme underweight In the marasmic case calculation of protein intake on a weight basis according to the estimated requirements of a child of this weight may result in an absurdly small total protein intake while calculation on the basis of chronological age as given in most tables of requirements (British Medical Association 1950 Canadian Council on Nutrition, 1950 National Research Council, 1953 Institute of Nutrition of Central America and Panama 1953) results in an impossibly high one The present recommendations for the 3 to 7 g per kg in the treatment of kwashiorkor attempt to take these factors into consideration and leave considerable room for judgement as to whether the lower or higher intakes within this range are most appropriate in a specific case If the total protein intake based initially on 5 to 7 g of protein per kg is kept constant the increasing weight of the recovering child results in a gradual reduction of the protein intake per kg to the 3 to 5 g per kg range and eventually less This is frequently the natural course of events since the child s willingness to eat and capacity to utilize large quantities of food decreases gradually as consolidation of cure approaches completion.

(b) *Proteins of Animal Origin*

Milk has been generally recommended as the most convenient source of protein for the treatment of kwashiorkor Skimmed milk has been widely used because it has proportionally more protein and less fat than whole milk and is recommended by many authors (Brock and Autret, 1952 DeMaeyer 1954 Dean, 1953 Trowell, 1954 Raoult, 1954 Silva 1954) as the most effective and practical way of administer





FIG 4 1 THIRTEEN MONTH-OLD BOY WITH CLASSICAL KWASHIORKOR (SUGAR BABY) ON ADMISSION (ABOVE) AND 6 WEEKS AFTER TREATMENT (BELOW)

Notice the abundance of subcutaneous fat and the swiftness of recovery

(*Composite photograph from Plates X and XI of the a ticle Kwashiorkor and Marasmus in Jamaican Infants by Jelliffe D B Bras G and Stuart K L in the West Indian Medical Journal 3 43 1954 Use of this illustration was kindly authori ed by Dr Jelliffe*)



FIG 4 2 BOY 3 YEARS 9 MONTHS OF AGE WITH MARASMIC KWASHIORKOR AS USUALLY OBSERVED IN CENTRAL AMERICA

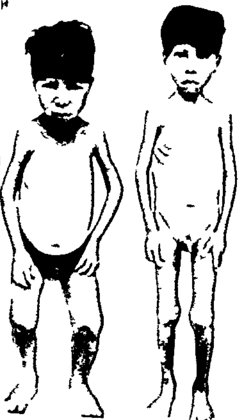


Fig 4 3 Boy 8 Years 11 Months of Age with Marasmic Kwashiorkor on Admission (Left) and 10 Days after Treatment (Right)

Notice how the loss of oedema makes more apparent the underlying wasting

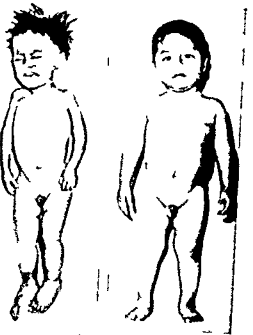


Fig 4 4 Boy 2 Years 8 Months of Age with Kwashiorkor on Admission (Left) and 19 Weeks after Treatment (Right) with INCAP Vegetable Mixture 8 as the Only Source of Protein

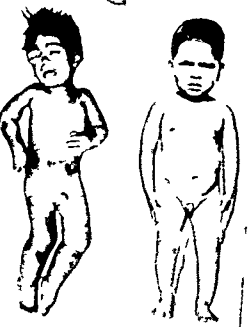


Fig 4 5 Boy 2 Years 8 Months of Age with Kwashiorkor on Admission (Left) and 15 Weeks after Dietary Treatment (Right) Initiated with Milk

ing the proteins needed in the therapy of kwashiorkor half skimmed milk, however has given equally good results (Béhar Viteri and Scrimshaw 1957 Béhar *et al* 1958) Some workers (Achar and Benjamin 1951 Gerbasi 1956) prefer an acidified half skimmed milk in the initial stages of the treatment believing it to be more easily digestible in the presence of the gastric hypochlorhydria and initial enzyme deficiencies of kwashiorkor (Veghelyi 1948 Gómez *et al* 1954*a* Badr El Din and Aboul Wafa 1957) Whole milk has also been used successfully from the beginning of treatment (Gomez *et al* 1958) although there are some reports of intolerance to it (DeMaeyer 1954, Dean 1953 Trowell 1954) Both Dean (1953) and DeMaeyer (1954) have recommended the addition of calcium caseinate (Casilan Casec) to milk to augment the protein content even more and state that this addition has also proved beneficial for the diarrhoea almost always present in these children at the time of hospitalization

It is universally recognized therefore that milk proteins constitute an excellent treatment of kwashiorkor The form in which they are administered is not important as long as the anorexia and inability to tolerate large quantities of food which are common in the acutely ill child are considered Good results can be achieved with any of the recommended milks through the use of proper dilutions and dosages We have achieved equally good results using either skimmed milk, half skimmed milk or acidified half skimmed milk Similarly Brock *et al* (1955) have not found any difference in effectiveness between skimmed milk and casein The possible influence of the quality and quantity of the fat and carbohydrate components of milk will be considered later

The view that milk owes its initial therapeutic efficacy entirely to its protein content is strongly supported by the success of Brock *et al* (1955) and Hansen Howe and Brock (1956) in initiating cure of kwashiorkor with vitamin free casein or even with suitable mixtures of synthetic amino acids They are able to bring about disappearance or marked improvement of all the clinical characteristics of kwashiorkor with these preparations although as they point out the other essential nutrients are soon needed for continued recovery

Other proteins of animal origin (meat eggs etc) have not been used in the initial stages of the treatment of kwashiorkor mainly because of the inherent difficulties in the administration of these products to the anorexic child It is recommended nevertheless that they be added as soon as convenient after the initial anorexia has disappeared. We have usually found it possible to introduce them not later than the end of the second or during the third week of hospitalization even in severe cases

(c) *Proteins of Vegetable Origin*

Proteins of vegetable origin have been employed in the treatment of kwashiorkor in some areas not in the expectation of obtaining better results than with milk whose value and convenience are undisputed but in an effort to assay their biological value as alternative protein sources for prevention Suitable vegetable protein sources are required since milk supplies are often inadequate in the underdeveloped areas of the world in which protein malnutrition is prevalent particularly for the vulnerable socio-economic groups in which kwashiorkor occurs

Gomez *et al* (1952) compared the progress of malnourished children treated with either a soya preparation or cow s milk and were unable to detect any differences in recovery by the clinical and biochemical criteria employed except for a slower rise in total serum protein the final serum protein levels in the two groups were identical Dean (1953) reported satisfactory results in the treatment of kwashiorkor with a soya preparation although both the disappearance of the oedema and the rise of serum albumin occurred at a somewhat slower rate than in similar cases treated with skimmed milk The more extensive trials of the treatment of kwashiorkor with proteins of vegetable origin carried out in India (Venkatachalam *et al* 1956) and Central America (Scrimshaw *et al* 1957*a*) have evaluated vegetable protein sources for both treatment and prevention In India 59 children were treated with a diet based on Bengal gram as the only source of protein another group of 19 similar children received their proteins from Bengal gram and rice Forty nine additional children receiving a skimmed milk diet of equal protein content served as controls The results of the treatment with proteins of vegetable origin were considered satisfactory although the disappearance of oedema and the rise of serum albumin again were slower than with milk this was particularly true for the Bengal gram–rice diet Although these findings suggest that the amino acid balance was not optimal in the vegetable protein diets employed, the skin lesions and the fatty infiltration of the liver disappeared as rapidly as with milk and no differences were observed in the rates of haemoglobin regeneration.

In Central America we have worked with two formulae based on plant products which are or can be made locally available INCAP Vegetable Mixture 8 contains 50 per cent dried corn masa, 35 per cent sesame meal, 9 per cent cottonseed press-cake 3 per cent torula yeast and 3 per cent kikuyu leaf meal (Scrimshaw *et al* 1957*b*) This preparation has been given to children with moderate to severe kwashiorkor and has resulted in uniformly good recoveries without complications (Fig 4 4) INCAP Vegetable Mixture 9 contains 28 per cent corn masa 28 per cent sorghum

masa 38 per cent cottonseed press-cake 3 per cent torula yeast and 3 per cent kikuyu leaf meal and is more economical to produce in Central America in the present circumstances Preliminary indications are that this formula also gives excellent results

The development of vegetable mixtures will be discussed in more detail under prevention since this is the primary purpose for which they have been developed Although the foregoing results leave no doubt that if necessary, proteins of vegetable origin can be successfully employed in the treatment of kwashiorkor the vegetable protein sources for this purpose must be carefully chosen and skilfully combined Their amino acid patterns must complement one another to correct the unbalanced pattern of amino acids characteristic to a greater or lesser degree of all vegetable proteins from a single food source

2. Caloric Intake

The caloric intake is of secondary importance in the initial stages of treatment although the diet should contain sufficient calories to ensure good protein utilization It is extremely difficult however to begin treatment with a diet supplying the desired caloric content owing to the anorexia and food intolerance of the child with acute kwashiorkor Accordingly we may give as few as 30 to 50 cal per kg during the first day and raise this level gradually to 100 cal per kg by the fifth or sixth day of therapy Although this caloric intake seems low for severely malnourished children it will ensure sufficient nitrogen retention to initiate recovery This finding agrees with observations of Gómez *et al* (1957*a*) which seem to indicate that with severe protein depletion nitrogen retention is still possible even in the presence of a low caloric intake when sufficient protein is given

As soon as possible after anorexia has disappeared, and the digestive enzymatic activity has been restored most authors recommend that the caloric intake should be increased gradually to about 150 cal per kg This can be reached toward the end of the first week or during the second week of treatment (DeMaeyer 1954 Dean 1953) An increase in caloric intake is necessary for good weight gain and satisfactory recovery after the loss of oedemas and during that part of recovery which Brock calls the consolidation of cure (Brock *et al* 1955) This is the variable period after disappearance of the acute signs and symptoms during which the child is gaining weight and gradually becoming ready for discharge from the hospital A high caloric intake is particularly important when the child is suffering from the marasmic type of kwashiorkor and is very much under weight after the loss of oedema.

Gómez *et al* (1958) have given as many as 200 to 250 cal per kg to children recovering from marasmic kwashiorkor It is probable however, that diets with such a high caloric content of predominantly carbohydrate origin are largely responsible for the nutritional recovery syndrome described by these authors. The symptoms which include enlargement of the liver ascites increased superficial circulation over the abdomen and hypertrichosis have not been reported with comparable frequency or severity by other workers although we have occasionally noted these symptoms in milder form in children of the marasmic type recovering on very ample diets This syndrome would appear comparable to that observed with the rapid refeeding of persons in prison and concentration camps following World War II and it should be regarded tentatively at least, as an indication that the upper limits of refeeding are being exceeded

3 Sources of Carbohydrate

Some authors have suggested that the child with kwashiorkor has a decreased tolerance for certain sugars especially lactose and sucrose (DeMaeyer 1954 Dean 1953, Trowell, 1954 Dean 1956) Dean (1953 1956) and DeMaeyer (1954) for example, believe that the high lactose content of skimmed milk may aggravate the tendency to diarrhoea and recommend the use of bananas and rice as carbohydrate sources in the initial phase of treatment Nevertheless most workers consider milk with added sucrose entirely satisfactory and in using acidified half skimmed milk we have not encountered any difficulties Acidified milk does contain less lactose than non acidified milk since part of the sugar is transformed into lactic acid but there is no evidence that this particular change is of significance In common with other investigators we find bananas to be well tolerated even as part of the initial treatment and use them regularly as a carbohydrate supplement.

The vegetable mixtures developed as protein sources contain carbohydrate in a very satisfactory form for the treatment of kwashiorkor The tendency for diarrhoea to disappear more quickly with the use of these vegetable mixtures than with milk has been repeatedly noted (Venkatachalam *et al* 1956 Gopalan 1956) and, as Dean (1953) has suggested may be due to the differences in carbohydrate content of the two types of dietary regimens

4 Amount and Type of Fat

The proper amount and kind of fat to be included in diets for the treatment of kwashiorkor especially during the early stages of recovery is still a matter of controversy There is little doubt that the ability to absorb fat and fat-soluble factors is initially impaired but this seems to be recovered very rapidly when adequate protein is supplied. The evidence for this

comes directly from studies of fat (Holemans and Lambrechts 1955 Gómez *et al* 1956) and of vitamin A absorption (Arroyave *et al* 1959) and indirectly from measurements of the lipase activity in the duodenum (Véghelyi 1948) Recovery of ability to absorb oral vitamin A occurs in 3 to 5 days with optimum dietary treatment (Arroyave *et al* 1959) and intestinal enzyme activity is restored within 3 days (Veghelyi 1948)

Certainly early in treatment fat absorption becomes proportional to fat intake (Holemans and Lambrechts 1955 Gómez *et al* 1956 Robinson *et al* 1957) Furthermore as Holt (1956) has strongly emphasized the presence of excessive fat in diarrhoeal stools in the initial stages of treatment is not a contraindication to the incorporation of fat in the diet While adding fat to the diet may increase faecal fat in this circumstance it also increases fat absorption so that useful additional calories are provided which spare protein and facilitate recovery

For these reasons we cannot accept the point of view that children with kwashiorkor cannot tolerate fat of any kind (Trowell, 1954) nor that milk fat should not be given (Dean 1953) We are impressed however with the success of Dean and Skinner (1957) in feeding 30 to 50 g of vegetable fat from soya or cottonseed They report that this quantity is well tolerated and aids greatly in maintaining adequate caloric intake The INCAP Vegetable Mixture 8 which has proved to be well tolerated even in severe cases of kwashiorkor contains 35 per cent of sesame oil meal with a fat content of 33 per cent and thus supplies about 20 g of fat from this source as fed to a 10 kg child by the end of the fifth day of treatment

Fat is not only a valuable source of calories but also provides essential fatty acids which may be of benefit to the child particularly since the characteristic diets leading to the development of kwashiorkor are often almost wholly devoid of fat In general it does not seem justifiable to restrict fat intake with the possible exception of the first two or three days of treatment

While we prefer to begin treatment with only the fat supplied by half skimmed milk whole milk is substituted by the end of the first week and additional fat of vegetable origin added to the diet so that 15 to 20 per cent of the total calories in the therapeutic diet are supplied by fat

5 Vitamin Requirements

The associated vitamin deficiencies in kwashiorkor vary in nature and severity from one region to another depending upon the diets which the children receive prior to developing the disease Vitamin A and some of the B-complex vitamins are particularly likely to be deficient because the diets in underdeveloped areas in general frequently result in these deficiencies In Central America lip and tongue changes suggestive of riboflavin deficiency are frequent in the general lower income groups (Perez, Arce Paiz and Maza, 1955) Studies carried out at INCAP on pre school children have also revealed the serum levels (Carrascosa 1956) and urinary excretion of this vitamin to be reduced (Arroyave Sandstead and Schumacher 1958)

The fact that vitamin A deficiency is observed even in regions where the dietary intake of this nutrient is not excessively low has led Trowell Moore and Sharman (1954) to suggest that the deficiency of vitamin A is due largely to the poor fat absorption When both low intakes and poor absorption occur avitaminosis A may be of such magnitude as to produce severe ocular lesions (Oomen 1954) such as Bitot s spots and keratomalacia In Central America eye lesions are frequent but usually not severe They include dryness of the cornea and thickening of the conjunctiva Serum levels of vitamin A and carotene however are nearly always very low (Scrimshaw *et al* 1956 Behar *et al* 1956 Scrimshaw *et al* 1955)

Ascorbic acid deficiency in kwashiorkor has been reported only occasionally since most underdeveloped areas have abundant natural sources of this vitamin In Central America we have encountered no clinical evidence of ascorbic acid deficiency serum levels of this vitamin are relatively high in most individuals Although we frequently find low serum levels of this vitamin in kwashiorkor they are not at the zero or near zero limits associated with clinical scurvy

Because signs suggestive of vitamin deficiency and skin lesions of a pellagroid type are almost universally present it is not surprising that many early investigators tried the administration of therapeutic doses of vitamins in the treatment of kwashiorkor Experience has demonstrated however that this is not only unnecessary but may prove detrimental Carvalho (1947) reports greater mortality in a group of children who were given B complex than in another group treated identically but without B complex supplement and attributes the higher mortality to a greater fatty infiltration of the liver Furthermore Brock *et al* (1955) have demonstrated that initiation of cure" that is the initial recovery which includes loss of oedema and apathy frank improvement of skin lesions recovery of appetite etc which usually occur before the twenty first day of treatment, is achieved with vitamin free casein and even with a mixture of synthetic amino acids

While some authors (DeMaeyer 1954 Gerbasi, 1956) routinely administer an oral preparation supplying physiological amounts of the common vitamins, this is not necessary for good recovery if attention is given to supplying all of the essential nutrients in a balanced diet. Trowell (1954) Altmann (1948) and

Gómez *et al* (1954*b*) report good recoveries without the use of any vitamin products We do not use them at all when employing vegetable mixtures which are balanced to contain sufficient vitamin A activity and B-complex vitamins When ocular lesions are present however and we are using half skimmed milk which fails to supply sufficient vitamin A we give 3 000 to 5 000 I U of vitamin A orally but only until adequate natural sources of this vitamin can be introduced into the diet

There is no evidence that a deficiency of vitamin B_{12} plays a role in kwashiorkor Even though the anaemia of kwashiorkor is frequently macrocytic in type (Scrimshaw *et al* 1956 Adams 1954 Mehta and Gopalan 1956) the bone marrow in Central America at least, is not megaloblastic Furthermore not only does this anaemia respond well to the administration of skimmed milk but the serum vitamin B_{12} values are within normal limits [1] Since megaloblastic anaemias have been reported in kwashiorkor (Adams 1954, Mehta and Gopalan, 1956) it is possible that in these cases vitamin B_{12} deficiency is superimposed on the primary protein deficiency of kwashiorkor as may occur with other vitamins of the B complex Dean (1953) has recommended massive doses of vitamin B_{12} when weight gain appeared stationary during the consolidation of cure Other authors have found no response to the administration of this vitamin (Trowell, 1954 Silva 1954) We have been unable to confirm the claim of benefit from vitamin B_{12} administration and have markedly reduced the frequency and duration of stationary periods in weight gain during recovery by taking precautions to reduce the spread of intercurrent infections, particularly that of diarrhoea of infectious origin

6 Mineral Needs

The characteristic anaemia of kwashiorkor is normocytic or macrocytic in type and generally mild unless complicating factors are present In regions where chronic malaria or hookworm occurs in young children severe microcytic hypochromic anaemia may be present initially and require specific iron therapy as part of the initial treatment Even when the anaemia is not of the iron-deficiency type it must be recognized that the stores of body iron are limited With a therapeutic diet based on milk there is a rapid haematopoiesis, without an iron intake adequate to meet the increased iron requirements The child may then develop a microcytosis and a decreased mean corpuscular haemoglobin concentration despite a continuing rise in the red blood-cell count In these circumstances iron has become a limiting factor and the administration of supplementary iron will evoke a second reticulocyte response and the gradual return of the mean corpuscular volume to normal.

Accordingly we consider it desirable to begin the administration of an iron preparation during the second or third week of treatment and have obtained good results with the oral administration of 300 to 600 mg of ferrous sulphate per day in single or divided doses As other investigators have also reported (De Maeyer 1954) such a preparation can be safely administered at this stage of recovery without intolerance

7 Other Dietary Supplements

In view of the fatty infiltration of the liver some early reports recommended the use of lipotropic agents (Meneghello 1949) However Carvalho (1947) does not find products such as choline chloride or desiccated pancreas to be useful, and most investigators now believe that the fatty infiltration of the liver in kwashiorkor is not related to a deficiency of lipotropic factors Particularly since this infiltration recedes quickly and completely upon administering proteins of a high biological value the use of lipotropic preparations does not appear justifiable

8 Summary of the INCAP Dietary Regimen

Although a variety of protein and caloric sources can be successfully employed we find it most convenient to begin the treatment of severe kwashiorkor cases with acidified half-skimmed milk diluted to half strength with water to which 5 per cent sugar has been added. Because of anorexia this is administered in doses of 120 cc every 2 hours (eight times a day) or if not well tolerated or accepted in this amount in smaller doses of 60 cc every hour (17 times a day) These quantities supply from 1 to 2 g of protein and from 30 to 60 cal per kg during the first 24 hours It may be given by gastric tube if necessary in extreme anorexia If it is well tolerated the concentration is increased to three-quarters strength during the second 24 hours and to full strength by the third or fourth day The amount of formula given is increased as rapidly as accepted to 180 cc every 3 hours (six times daily) and then to 240 cc at the same intervals (five times daily) so that the child receives approximately 5 g of protein and 100 cal per kg by the fourth to sixth day of hospitalization. We begin to give the child bananas as soon as he will accept solid food, usually on the second or third day after admission.

During the second week of therapy the child is gradually changed to whole non-acidified milk and the amount is increased to supply approximately 7 0 g of protein per kg. With two bananas a day and 120 cc

[1] The co-operation of Dr Grace Goldsmith, Professor of Medicine Tulane University School of Medicine in arranging for the vitamin B_{12} determinations is gratefully acknowledged.

of orange juice the total calories can be readily brought to approximately 130 per kg During the second and third week green and yellow vegetables are added to the diet and by the third or fourth week meat egg cereals and bread can all be given By the end of the first month the child should be receiving a varied and balanced diet supplying 5 to 6 g of protein per kg 140 to 150 cal per kg and containing all the necessary vitamins and minerals in physiological amounts No vitamin supplements need be administered An oral iron preparation supplying 60 to 120 mg of iron is introduced during the second to fourth week of treatment to meet the increased demand occasioned by rapid haematopoiesis (Fig 4 5)

B Correction of Electrolyte Disturbances

The treatment of kwashiorkor is greatly complicated by potassium depletion which is reported to be a manifestation of the severe protein deficiency (Hansen and Brock 1954 Metcoff *et al* 1956 Politzer and Wayburne 1957 Senecal 1958) and in most instances also by a serious fluid and electrolyte imbalance resulting from diarrhoea and occasionally from vomiting (Metcoff *et al* 1956 Gomez *et al* 1957b) Hansen and Brock (1954) consider that the potassium deficiency is a factor in the aetiopathogenesis of the oedema while Thompson (1955) reports hypokalaemia only in patients with diarrhoea upon admission but finds that even in those cases without diarrhoea a subsequent potassium deficiency can develop if the child is given a diet high in protein without sufficient potassium to maintain a normal nitrogen/potassium retention ratio Furthermore serum potassium levels do not always reflect the basically intracellular deficiency of this electrolyte Balance studies conducted by Hansen and Brock (1954) in South Africa and tissue analysis carried out in Mexico (Metcoff *et al* 1956 Frenk *et al* 1957) demonstrate a deficiency of potassium in kwashiorkor both determinations are much more revealing than the measurement of serum levels

Finally some of the clinical manifestations of acute kwashiorkor such as the psychic changes anorexia vomiting abdominal distention and weakness (Politzer and Wayburne 1957 Thompson 1955) may be explained at least in part by potassium deficiency

In order to correct this disturbance some authors recommend the routine oral administration of potassium at least in those cases in which a deficiency is suspected Thompson (1955) recommends 1 g daily of potassium chloride during the first 10 days similarly Politzer and Wayburne (1957) employ a mixture of equal parts of potassium acetate bitartrate and citrate and even recommend the intravenous administration of potassium chloride when there is evidence of hypokalaemia

Senecal (1958) recommends the addition of 100 to 200 mg of potassium per kg per day to the therapeutic diet preferably as citrate or acetate in order to avoid aggravating the acidosis resulting from a high protein diet

We have obtained good results with the addition of 1 g of potassium chloride to the diet in those cases suffering from persistent diarrhoea or which have clinical manifestations suggesting a potassium deficiency we do not prescribe it however until we are certain that diuresis is adequate Appreciable amounts of this element should not be given parenterally except under careful laboratory control of serum levels and adequate facilities to follow electrocardiographic changes

Serious disturbances in water and electrolyte balance have been recognized as important causes of mortality during the first forty-eight hours of hospitalization (Gómez *et al* 1957b) Frequently these children enter the hospital severely dehydrated as a result of prolonged diarrhoea and possibly also vomiting even though the severity of the dehydration may be partially concealed by the oedema Particularly detailed and valuable investigations of this problem have been carried out in the Children s Hospital of Mexico City (Metcoff *et al* 1956 Gomez *et al* 1957b Frenk *et al* 1957 Gordillo *et al* 1957 Metcoff *et al* 1957) The results of these studies indicate that the electrolyte disturbances in malnourished children with diarrhoea are qualitatively different from those of well nourished children with this symptom and much more difficult to correct

Two fundamental characteristics are reported for the dehydrated and severely malnourished children—(1) hypotonicity of the extracellular fluid with an expansion of the aqueous intracellular phase independently of the volume of extracellular fluid (2) an increase of intracellular sodium relative to the increased water content of this compartment and reduction of both the absolute and relative intracellular concentration of potassium The specific cause of these alterations is at present unknown but they are usually reversible with treatment Death sometimes occurs however as a direct consequence of the disproportionate expansion of intracellular at the expense of the extracellular fluid In malnourished children there is a decrease of the filtration rate through the kidneys considered by the Mexican workers to represent a defence mechanism for the maintenance of extracellular volume The renal plasma flow is also decreased and this is interpreted as an adaptation to the cellular hypotonicity

These alterations explain why many children do not respond to the measures usually employed to correct dehydration in a well nourished child The authors report the successful treatment of extremely severe cases with intravenous hypertonic saline for the

purpose of relieving the intracellular oedema However as the authors state, before recommending general use of this potentially dangerous measure additional studies and observations are necessary

To combat this electrolytic imbalance, Brock *et al* (1955) recommend *the oral administration, or intra*venous if necessary, of an electrolyte solution (such as Darrow s or Hartman s) with glucose during the first twelve to twenty four hours When there is no *clinical evidence of dehydration* we *follow* a similar practice

If there is severe dehydration associated with diarrhoea and/or vomiting we treat this condition and disregard the coexistent malnutrition and the presence of oedema. Usually treatment is initiated with the intravenous administration of the so-called 1–2–3 solution which contains 1 part of one sixth molar sodium lactate 2 parts of Ringer solution and 3 parts of 5 per cent glucose solution This is a hypotonic solution, and according to the data obtained from studies *carried out in Mexico, it should not be the* method of choice Nevertheless in contrast to the abundant hypotonic urinary excretion reported by workers in Mexico we have observed a marked oliguria in these children, and have found that both the dehydration and oliguria are considerably improved after the intravenous administration of hypotonic fluids in the amount of 40 to 50 cc per kg at a rate of 40 to 50 drops per minute Once we have obtained sufficient diuresis we continue with a solution richer in potassium (Darrow s) and if necessary, with additional amounts of Ringer or normal saline solution in 5 per cent glucose until the disorder has been corrected If vomiting has been too severe and alkalosis is suspected, as may occasionally be the case *the sodium lactate is omitted from the treatment* described

Obviously serial determinations of the child s ionogram can be *of invaluable help in deciding the* measures to be taken in the correction of the electrolytic imbalance This is at present impossible however in the majority of hospitals in the underdeveloped areas of the world where kwashiorkor is a frequent problem The indicated measures which have been published in detail previously (Béhar, Viteri and Scrimshaw 1957) have proved effective and easy to carry out even without laboratory facilities However we strongly urge further studies of the characteristics mechanism of production and methods of correcting electrolytic disorders which are commonly present in the child with kwashiorkor and appear to be a frequent and baffling cause of death.

C. Role of Blood Transfusions

Blood transfusions have long been used in the treatment of kwashiorkor (Carvalho, 1947 Meneghello 1949 Van der Sar, 1951 Gómez *et al.*, 1954c Autret and Béhar 1954, *Waterlow and Vergara*, 1956) Although in the past they have helped to compensate for inadequate initial therapy they are rarely necessary if good dietary treatment is given. *Only in the unusual case of* severe secondary anaemia or circulatory collapse and shock from either toxic infectious processes or very severe dehydration do we employ blood transfusions at present In the latter they are not given until after initial treatment with appropriate electrolyte solutions Small transfusions of from 10 to 20 cc per kg, which can be repeated if necessary are preferable since greater amounts given at one time may cause circulatory embarrassment.

Blood transfusions are a costly and ineffectual means of *administering protein* and should not be used for *this purpose, protein can be supplied* in adequate quantities by dietary means and the expense of transfusions for this purpose is totally unjustified. The needlessness of blood transfusion emphasizes *further the important fact that even severe kwashior*kor can be treated successfully in the small hospitals in rural areas with limited facilities and funds

D Use of Plasma and Protein Hydrolysates

Plasma should be used only when blood is not available for the treatment of the rare cases of circu*latory collapse due to secondary complications There* is no justification for the use of either plasma or parenteral protein hydrolysates as a protein source and there is evidence from refeeding of prisoners *after World War II that the latter may aggravate* liver damage in severe malnutrition

E. Need for Antibiotics and Chemotherapy

Every sign or symptom of infection observed in a child with kwashiorkor should be treated promptly *and energetically with antibiotics or chemotherapy since infections interfere* with the recovery of the child and may be more serious than they at first appear Even when signs of infection are not detectable at the time of admission we prefer to administer full therapeutic doses of *penicillin during the first 10 days of* hospitalization and find that this practice aids in the prevention of deaths that otherwise occur during the first week due to severe secondary infection. Bronchopneumonia, *for example may develop asymptomatically* without fever leucocytosis and even without any respiratory sign or symptom. The seriousness of the problem is illustrated by the fact that 70 per cent of all autopsies of *kwashiorkor deaths in Guatemala* reveal a significant degree of broncho-pneumonia (Tejada, Béhar and Cofiño 1956) and bronchopneumonia is a major terminal cause of death wherever kwashiorkor occurs For this reason the *administration*

of either antibiotics or sulphonamides is widely employed as a routine preventive measure (Hansen Howe and Brock 1956 Waterlow and Vergara 1956 Symonds and Mohammed 1956)

No specific treatment need be given for diarrhoea since this is present upon admission in almost every child with kwashiorkor and usually disappears after a few days of adequate dietary treatment When amoebae are identified in stool samples however specific therapy should be given promptly Similarly children in malaria regions should have their blood examined for malaria parasites and should be treated adequately if these are found Although intestinal helminths are very commonly present, no effort should be made to eliminate them until the child is well recovered They seldom interfere significantly with recovery and most antihelminthic drugs are dangerously toxic to severely malnourished children Considerable numbers of intestinal helminths particularly ascaris are often spontaneously expelled coincidently with the initial dietary treatment

F General Hospital Care

Children suffering from kwashiorkor require special care and treatment during the initial phases of recovery The attending staff must make sure that the child actually ingests the quantities of food ordered even if it is necessary to resort to tube feeding However with patient and responsible personnel gastric intubation can be avoided most of the time In studies of the recovery of vitamin A absorption ability in kwashiorkor (Arroyave *et al* 1959) we have observed that children admitted to a general hospital ward took more than twice as long to recover this function than children under research care even though identical orders for a therapeutic diet were written by the physician Careful investigation revealed that the attending personnel in the general hospital did not have the time or patience to feed the children the prescribed diet

In some hospitals mothers are encouraged to stay in the hospital and help with the care of their children (Geber and Dean, 1956) This has the double advantage of ensuring individual attention for the child and teaching the mother correct feeding practices For most mothers and hospitals this may be impractical but frequent visits by the mother make possible careful explanation to her of the treatment given and the reasons why her child developed the disease Since ignorance of proper feeding of young children is a major factor in the development of kwashiorkor this type of instruction is often very effective In follow up visits to families with a child who has been treated for kwashiorkor we sometimes find the former patient in superior physical condition to others in the neighbourhood because the parents have learned the importance of good nutrition

The cross infections common to a general hospital ward also greatly interfere with normal recovery and prolong hospitalization time The treatment of kwashiorkor under conditions of semi isolation instead of an open ward has been a major factor in reducing our usual hospitalization from 16–20 weeks to 10–14 weeks The cost of separating children with kwashiorkor from each other and from those with general paediatric conditions is more than met by the saving due to the shorter hospital stay More space screens between beds and more care in the washing of hands after attending other children with diarrhoea are helpful and individual cubicles are desirable These should be combined with hygienic measures on the part of personnel to limit cross-infections

In severe cases with marked apathy and muscular hypotonicity oedemas or skin exulcerations frequent changes in position and cleanliness are particularly necessary in order to avoid decubitus ulcers or secondary infections of the cutaneous lesions

It is also important to recognize that the children do react favourably to sympathy and kindness on the part of the personnel. Some children respond so remarkably to affectionate attention in the hospital as to suggest that its lack may have been a factor in the development of the syndrome as postulated by Geber and Dean (1956) for many African cases Certainly it is particularly important that kwashiorkor cases be placed in the care of attendants who like children and are patient with them.

IV FACTORS RESPONSIBLE FOR THE DEVELOPMENT OF CLINICAL CASES

Any discussion of the factors responsible for kwashiorkor must emphasize that clinical kwashiorkor *per se* is only an indication of the total extent of protein malnutrition among children in endemic areas In most regions in which kwashiorkor is a public health problem, nearly all young children are affected by protein malnutrition even though they may never develop the fully fledged syndrome Very often they do not even present sufficient obvious signs or symptoms to be considered ill (Scrimshaw *et al* 1955 1957*a*) but their sub-optimal status is objectively revealed by retarded growth and development which begins about the eighth month of life The retardation is evident first in failure to register normal gains in weight and later in height as well as bone maturation, as compared with well-nourished children of the same age This retardation is the result of inadequate supplementary feeding when the mother's milk, which

previously had maintained the child in a good nutritional state becomes insufficient to satisfy physiological requirements and later is withdrawn entirely as the result of weaning Both growth and development may remain practically at a standstill until the child is three to five years old After this depending on the local circumstances he receives relatively better nourishment which is more nearly that of the adult at the same time his requirements per kg of body weight gradually decrease

Children suffering from sub-clinical protein deficiency tend to be apathetic show marked muscular weakness and wasting and are slower to mature They become ill easily and very often die of an infectious disease that is usually not fatal to well nourished children Even with very inadequate diets the physical examination may not reveal any signs of nutritional deficiency other than retardation of growth and maturation

The problem of preventing kwashiorkor therefore consists not only in the elimination of the syndrome in its frank form but also in the correction of the underlying malnutrition affecting the great majority of children in some areas thus the presence of kwashiorkor must be considered only as the visible reminder of a grave and largely hidden problem

While the prevention of kwashiorkor requires adequate food which in turn involves economic factors limited purchasing power is only one aspect and not usually the principal one Diets consumed by school children and adults even in the poorest communities are usually not as deficient in relation to physiological requirements for protein as those consumed by pre school children In many regions of the world the majority of these children are breast fed until they are at least one year old and frequently especially in the rural areas until they are one and a half or two years old or even longer In present circumstances this prolonged breast feeding is advantageous for the child who after weaning receives a diet markedly deficient in protein particularly in protein of animal origin Supplementary feeding is introduced late to nursing infants generally after the eighth or ninth month and often after the child is one year old The foods employed in the supplementary feeding are the same foods given to the child after weaning Unfortunately the mother tends to consider that foods easily swallowed by the child are also easily digested Accordingly toward the end of breast feeding and after weaning the child s diet is based primarily on starchy gruels thin broths breads rice noodles a few vegetables and coffee Although beans, for example constitute a rich source of protein in the adult diet in Central America small children are ordinarily given only the water in which these beans are cooked When the family can afford meat it is consumed only by the adults and older children for it is not considered appropriate for the small child.

The foregoing are merely examples of the great influence of ignorance on the prevalence of kwashiorkor With local variations and in greater or lesser degree poor feeding practices for the young child exist in all areas where kwashiorkor is prevalent (Williams 1954 Jelliffe 1955) Unfortunately some prejudices against particular foods have a basis in experience and are accordingly difficult to combat Prejudices against milk are an example They exist in part because of the poor sanitary conditions under which milk has been available and its consequent association with diarrhoeal disease and even with death Thus when the mothers are told that cow s milk is a valuable food for the small child they may still be reluctant to give it to their children even when it is made available Ignorance and lack of facilities for adequate preservation may also result in good milk supplies becoming unfit for consumption by the time they reach the child

Prejudices such as the one described have particularly serious consequences when the child becomes ill Feeding is restricted even more in the presence of any sickness and especially when the child has diarrhoea The mothers believe that in order to correct the diarrhoea the child should be placed on a diet restricted largely to starchy gruels rice water or sugar water Such diets are sometimes prolonged for several weeks since the child fails to improve and the result is clinical kwashiorkor and death Unfortunately some physicians still recommend this type of diet in cases of diarrhoea in small children and thus help to perpetuate the belief and practice

It is no coincidence that most kwashiorkor cases in Guatemala give a history of an episode of diarrhoea of apparently infectious origin shortly before the onset of the oedema skin lesions and other signs of kwashiorkor (Scrimshaw *et al* 1957*a*) In addition to the worsening of the diet as the result of misguided therapeutic efforts there is a direct contribution of the diarrhoea to protein malnutrition Even with mild and transient diarrhoea and a relatively high protein intake of good quality it has been shown experimentally that nitrogen retention in young children may actually become negative with this type of gastro-intestinal disturbance (Robinson *et al* 1957 Macy 1958) and remain markedly reduced for a period of days thereafter But the infectious diarrhoea affecting malnourished children in most technically underdeveloped areas is neither mild nor transient It is sufficiently severe and frequent as to be a major primary cause of death among children under five years of age (Verhoestraete 1956 Verhoestraete and Puffer 1957) Preliminary studies in rural Guatemala conducted by the Institute of Nutrition of Central America and

Panama (INCAP) indicate that at least 5 per cent of all children between one and four years of age have clinical diarrhoea at any given time in two moderately poor small highland towns house to house visits every two weeks for twelve months revealed an average of five separate episodes of diarrhoea per year for children in the same age range Prevalence studies carried out in four highland and four lowland towns over a two-year period suggest that much of the enteric infection is due to various strains of Shigella at least as evidenced from even a single faecal examination by means of the rectal swab technique (Watt and Hardy 1945 Hardy and Watt 1948 Watt *et al* 1953 Hollister *et al* 1955) Shigellas were recovered in nearly 8 per cent of all children under ten years of age examined (Beck Muñoz and Scrimshaw 1957) while Salmonellas were present in less than 1 per cent In most children from areas where kwashiorkor is endemic, there is also a very high prevalence of intestinal parasites of several species While they do not seem to be factors of primary importance intestinal parasites can have an adverse effect on protein absorption (Venkatachalam and Patwardhan 1953)

Not only intestinal infections but also systemic ones can apparently have a markedly adverse effect on nitrogen retention We were recently astonished to find that even during the prodromal stage of measles nitrogen retention was reduced in two children recovering from kwashiorkor This would explain why there are children with histories of measles chicken pox or other systemic infection which closely precedes the development of kwashiorkor For children who are already basically malnourished the added stress of infection enteric or systemic is often the factor determining the actual onset of acute kwashiorkor

Kwashiorkor arises out of ignorance of proper feeding practices for the young child a high prevalence of enteric infections due to poor sanitary practices prejudices against the use of milk and other protein containing foods of animal origin for young children limitations in food production due to poor agricultural practices and low purchasing power of the family as part of general poverty All of these with the possible exception of the last are subject to direct attack by health workers teachers and agriculturists

The methods and opportunities for so doing are discussed in the sections which follow and although they are presented with special reference to protein deficiency they are generally applicable to the nutritional problems of children in technically underdeveloped areas Techniques for the direct improvement of the economic status of countries or peoples are however considered to be beyond the scope of this review

V INCREASING PROTEIN SUPPLIES FOR HUMAN CONSUMPTION

The prevention of protein malnutrition requires greater availability and use of foods or combinations of foods whose protein is of good biological value It is axiomatic that for good health and normal growth and development food must do more than satisfy hunger and provide energy it must furnish protein containing the essential amino acids in the amounts and proportions required by the body supplying also the other essential nutriments It is appropriate therefore to examine ways in which the quantity and quality of protein for human consumption in technically underdeveloped areas may be increased

A. Meat and Dairy Products

Products of animal origin are the major source of protein of high biological value in more technically developed countries For young children cow s milk is of greatest importance The capacities of technically underdeveloped areas to develop an animal industry without undue competition with food crops are often underestimated. Most such regions contain large areas which are suitable only for raising live stock and should be utilized for this purpose Further more farm animals, particularly ruminants, can thrive on foods and by products which are not ordinarily suitable for human consumption. There have also been great technological advances in the feeding and management of livestock in tropical areas which should be applied Some of these measures such as the introduction of new grasses and improved management of grasslands the control of ecto- and endoparasites the maintenance of dry season production through the use of silage and artificial feeding and the use of proper mineral supplements can revolutionize tropical agriculture and result in large increases in the availability of animal protein in areas in which kwashiorkor is now prevalent

For example in nearly all underdeveloped areas of the world better management of cattle and the use of forages of high nutritional quality would significantly contribute to increase the production of animal proteins particularly milk and milk products and meat It has been shown in the American tropics that the production of animal products can be increased by the production and use of forages of good quality such as Desmodium (*Desmodium intortum*) kikuyu grass (*Pennisetum clandestinum*) quinamul (*Ipomonea sagittata*) ramie (*Boehmeria nivea*) and others (Squibb *et al* 1952 Squibb *et al.*, 1954 Bressani Elias and Jarquin, 1959)

The production of another important animal protein source poultry and eggs, could also be greatly

previously had maintained the child in a good nutri tional state becomes insufficient to satisfy physiological requirements and later is withdrawn entirely as the result of weaning Both growth and development may remain practically at a standstill until the child is three to five years old After this depending on the local circumstances he receives relatively better nourishment which is more nearly that of the adult at the same time his requirements per kg of body weight gradually decrease

Children suffering from sub-clinical protein deficiency tend to be apathetic, show marked muscular weakness and wasting and are slower to mature They become ill easily and very often die of an infectious disease that is usually not fatal to well nourished children Even with very inadequate diets the physical examination may not reveal any signs of nutritional deficiency other than retardation of growth and maturation

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While the prevention of kwashiorkor requires adequate food which in turn involves economic factors limited purchasing power is only one aspect and not usually the principal one Diets consumed by school children and adults even in the poorest communities are usually not as deficient in relation to physiological requirements for protein as those consumed by pre school children In many regions of the world the majority of these children are breast fed until they are at least one year old and frequently especially in the rural areas until they are one and a half or two years old or even longer In present circumstances this prolonged breast feeding is advantageous for the child who after weaning receives a diet markedly deficient in protein particularly in protein of animal origin Supplementary feeding is introduced late to nursing infants generally after the eighth or ninth month and often after the child is one year old The foods employed in the supplementary feeding are the same foods given to the child after weaning Unfortunately the mother tends to consider that foods easily swallowed by the child are also easily digested Accordingly toward the end of breast feeding and after weaning, the child s diet is based primarily on starchy gruels thin broths breads, rice noodles a few vegetables and coffee Although beans, for example constitute *a rich source of protein in the adult diet* in Central America, small children are ordinarily given only the water in which these beans are cooked When the family can afford meat, it is consumed only by the adults and older children for it is not considered appropriate for the small child

The foregoing are merely examples of the great influence of ignorance on the prevalence of kwashiorkor With local variations and in greater or lesser degree poor feeding practices for the young child exist in all areas where kwashiorkor is prevalent (Williams 1954 Jelliffe 1955) Unfortunately some prejudices against particular foods have a basis in experience and are accordingly difficult to combat. *Prejudices against milk are an example They exist in* part because of the poor sanitary conditions under which milk has been available and its consequent association with diarrhoeal disease and even with death Thus when the mothers are told that cow s milk is a valuable food for the small child they may still be reluctant to give it to their children even when it is made available Ignorance and lack of facilities for adequate preservation may also result in good milk supplies becoming unfit for consumption by the time they reach the child

Prejudices such as the one described have particularly serious consequences when the child becomes ill Feeding is restricted even more in the presence of any sickness and especially when the child has diarrhoea The mothers believe that in order to correct the diarrhoea the child should be placed on a diet restricted largely to starchy gruels rice water or sugar water Such diets are sometimes prolonged for several weeks since the child fails to improve and the result is clinical kwashiorkor and death Unfortunately some physicians *still* recommend *this type of diet in* cases *of* diarrhoea in small children and thus help to perpetuate the belief and practice

It is no coincidence that most kwashiorkor cases in Guatemala give a history of an episode of diarrhoea of apparently infectious origin shortly before the onset of the oedema skin lesions and other signs of kwashiorkor (Scrimshaw *et al* 1957a) In addition to the worsening of the diet as the result of misguided therapeutic efforts there is a direct contribution of the diarrhoea to protein malnutrition. Even with mild and transient diarrhoea and a relatively high protein intake of good quality it has been shown experimentally that nitrogen retention in young children may actually become negative with this type of gastro-intestinal disturbance (Robinson *et al* 1957 Macy 1958) and remain markedly reduced for a period of days thereafter But the infectious diarrhoea affecting malnourished children in most technically underdeveloped areas is neither mild nor transient It is sufficiently severe and frequent as to be a major primary cause of *death among children under five years of age* (Verhoestraete 1956 Verhoestraete and Puffer 1957) Preliminary studies in rural Guatemala, conducted by the Institute of Nutrition of Central America and

Panama (INCAP) indicate that at least 5 per cent of all children between one and four years of age have clinical diarrhoea at any given time in two moderately poor small highland towns house-to-house visits every two weeks for twelve months revealed an average of five separate episodes of diarrhoea per year for children in the same age range Prevalence studies carried out in four highland and four lowland towns over a two-year period suggest that much of the enteric infection is due to various strains of Shigella at least as evidenced from even a single faecal examination by means of the rectal swab technique (Watt and Hardy 1945 Hardy and Watt 1948 Watt *et al* 1953 Hollister *et al* 1955) Shigellas were recovered in nearly 8 per cent of all children under ten years of age examined (Beck, Munoz and Scrimshaw 1957) while Salmonellas were present in less than 1 per cent In most children from areas where kwashiorkor is endemic there is also a very high prevalence of intestinal parasites of several species While they do not seem to be factors of primary importance intestinal parasites can have an adverse effect on protein absorption (Venkatachalam and Patwardhan 1953)

Not only intestinal infections but also systemic ones can apparently have a markedly adverse effect on nitrogen retention We were recently astonished to find that even during the prodromal stage of measles nitrogen retention was reduced in two children recovering from kwashiorkor This would explain why there are children with histories of measles chicken pox or other systemic infection which closely precedes the development of kwashiorkor For children who are already basically malnourished the added stress of infection enteric or systemic is often the factor determining the actual onset of acute kwashiorkor

Kwashiorkor arises out of ignorance of proper feeding practices for the young child a high prevalence of enteric infections due to poor sanitary practices prejudices against the use of milk and other protein containing foods of animal origin for young children limitations in food production due to poor agricultural practices and low purchasing power of the family as part of general poverty All of these with the possible exception of the last are subject to direct attack by health workers teachers and agriculturists

The methods and opportunities for so doing are discussed in the sections which follow and although they are presented with special reference to protein deficiency they are generally applicable to the nutritional problems of children in technically underdeveloped areas Techniques for the direct improvement of the economic status of countries or peoples are however considered to be beyond the scope of this review

V INCREASING PROTEIN SUPPLIES FOR HUMAN CONSUMPTION

The prevention of protein malnutrition requires greater availability and use of foods or combinations of foods whose protein is of good biological value It is axiomatic that for good health and normal growth and development food must do more than satisfy hunger and provide energy it must furnish protein containing the essential amino acids in the amounts and proportions required by the body supplying also the other essential nutriments It is appropriate therefore to examine ways in which the quantity and quality of protein for human consumption in technically underdeveloped areas may be increased

A. Meat and Dairy Products

Products of animal origin are the major source of protein of high biological value in more technically developed countries For young children cow's milk is of greatest importance The capacities of technically underdeveloped areas to develop an animal industry without undue competition with food crops are often underestimated Most such regions contain large areas which are suitable only for raising livestock and should be utilized for this purpose Furthermore farm animals particularly ruminants can thrive on foods and by products which are not ordinarily suitable for human consumption There have also been great technological advances in the feeding and management of livestock in tropical areas which should be applied Some of these measures such as the introduction of new grasses and improved management of grasslands the control of ecto- and endoparasites the maintenance of dry season production through the use of silage and artificial feeding and the use of proper mineral supplements can revolutionize tropical agriculture and result in large increases in the availability of animal protein in areas in which kwashiorkor is now prevalent

For example in nearly all underdeveloped areas of the world better management of cattle and the use of forages of high nutritional quality would significantly contribute to increase the production of animal proteins particularly milk and milk products and meat It has been shown in the American tropics that the production of animal products can be increased by the production and use of forages of good quality such as Desmodium (*Desmodium intortum*) kikuyu grass (*Pennisetum clandestinum*) ramie (*Boehmeria nivea*) quinamul (*Ipomonea sagittata*) and others (Squibb *et al* 1952 Squibb *et al* 1954 Bressani Elias and Jarquin 1959)

The production of another important animal protein source poultry and eggs could also be greatly

increased through better feeding, management and selection of the birds That this can be done even with locally compounded all vegetable rations has been demonstrated For example good-quality forages (Squibb Guzmán and Scrimshaw 1953) palm nut oil meals such as corozo mbocayá palm and African palm (Squibb and Wyld 1952 Squibb Aguirre and Bressani 1958) can be used successfully in poultry rations These by products of the oil industry cannot be used directly by humans because of their high fibre content which is uneconomical to remove

B Fish and Other Sea Food

Most countries have an opportunity to secure additional quantities of animal protein from fish and other sea food The food potential of various products of the sea has been discussed recently by Weiss (1953) who has presented an impressive series of illustrations of types of highly nutritious foodstuffs which could be harvested from the sea in large quantities He emphasizes that the food reserves of the sea molluscs crustaceans (and even seaweeds) as well as fish deserve careful research to determine the best means of utilizing them In addition to marine fisheries the culture of fish in natural or artificial fish ponds is already widely practised in the orient and can be adapted to most tropical regions

The overall world shortage of protein of good quality has already stimulated studies on the nutritive value of derived products such as fish flour Great uniformity has been observed in the amino acid composition of the protein of fish the amino acid proportions of which are very similar to those of casein (Jaffe *et al* 1957) Fish proteins like other animal proteins are excellent sources of lysine methionine and tryptophan amino acids usually deficient in the cereal proteins which are the staple foods in underdeveloped areas (Flores and Reh 1955a b c 1955d F A.O 1956 Sebrell and Hand 1957) On the other hand, the biological value of the protein in derived processed fish products may differ Bender and Haizelden (1957) studied twenty seven fish meals and deodorized fish flours intended for human consumption and found net protein utilizations ranging from 18 to 80 per cent digestibilities from 47 to 97 per cent and biological values from 36 to 82 per cent This and other studies (Cravioto *et al* 1955 Carpenter *et al.*, 1957) demonstrate that it cannot be assumed that a fish flour is a good protein source for human use until biological trials are carried out

C. Legumes

The relatively high protein content and good protein quality for a single vegetable source the soybean, has been extensively demonstrated and reviewed (Payne and Stuart, 1944 Cahill, Schroeder and Smith, 1944) In some areas soya is already a protein source of major importance to the human population, and wherever it is feasible to introduce or increase the production and consumption of soya and soya products the problem of providing adequate protein supplies is greatly simplified It should be noted, however that it is not always easy to secure its acceptance by populations not accustomed to its use and that the agronomic details of growing it have not been worked out for many of the regions in which protein malnutrition is a serious problem For these areas it represents a potential long range rather than an immediately applicable contribution to the solution of the problem

Another promising protein source is peanut meal Although it has the present disadvantage of high cost for many needy regions its nutritive value alone and in combination with other foodstuffs has been well demonstrated (Sure 1948a Arthur, 1951 Holemans, Lambrechts and Martin 1956) It is regarded as a practical component of vegetable mixtures for child feeding in some areas and is under test in West Africa (Senecal 1958) In South Africa dehydrated cowpea flour is commercially available as a protein source for supplementary feeding, particularly for school lunches (Hansen 1958) The amino acid composition of a number of other tropical legumes has been studied (Vijayaraghavan and Srinivasan 1953 Baptist 1954 Jelliffe *et al* 1956) In general these plant protein sources have a fair amino acid composition and their use as foodstuffs should be increased in areas where diets are deficient in protein

D Oil Seed Meals

In addition to the introduction of protein rich plants such as soybean peanuts, and legumes the press-cakes of oil seeds can be utilized for human feeding This requires special processing as is the case with the low fibre low gossypol content cottonseed meal flour now available in the United States Because of the economic importance of cottonseed meal to the cotton industry as a by product and to the livestock industry as a protein supplement cottonseed products as foodstuffs have received considerable attention and reliable information is now available as to their elaboration for use in foods destined for human consumption.

Other oil seeds which are being tested as possible protein concentrates for use in child feeding, are rape seed (*Brassica napus*) sunflower seed (*Helianthus annuus*) and safflower seed (*Carthamus tinctorius*) (Fekete and Korpaczy 1955 Klain *et al* 1956 Hale and Brown, 1957 Petersen *et al.*, 1957) Rape seed and sunflower seed oil meals are high in fibre and low in lysine (Klain *et al.*, 1956) However Fekete and Korpaczy (1955) have described a specially prepared

sunflower seed oil meal with a biological value surpassing that of soya Decorticated safflower seed oil meal has been shown by Petersen *et al* (1957) to replace satisfactorily part of the soybean oil meal in poultry rations for both growth and egg production When substances toxic to animals occur in seeds detoxified products can be prepared by special chemical treatment For example Ambekar and Dole (1957) have recently described a practical method for detoxifying castor seed cake for use in animal feeding thus making available a protein rich material used before only as a fertilizer Thus all the materials described above could by the proper cultivation management and processing be developed into useful products for the feeding of animals in order to increase animal protein production and in combination with other foods for the feeding of humans

E. Other Seeds

In the last few years reports have appeared in the literature on the biological value of certain seed proteins which may be useful either alone or in combination with other sources of vegetable protein Sure (1955) found buckwheat flour (*Fagopyrum esculentum*) when given to rats at the 8 per cent protein level to have the highest biological value of any known plant source of protein The biological value of buckwheat protein for rats was 92 3 per cent of that of skimmed milk and 81 4 per cent of that of whole egg

Quinua seed (*Chenopodium quinoa* Willd) used for food in some South American countries has been shown by Quirós Perez and Elvehjem (1957) to have an excellent biological value as judged by the growth and lipotropic responses of weanling white rats Under the experimental conditions used at least seven essential amino acids had to be added to quinua to obtain slightly better weight gains than with quinua alone The authors showed that the addition of 3 per cent casein to quinua giving a total dietary protein of 12 42 per cent produced the excellent growth of 36 7 g per rat per week and a normal amount of liver fat This study confirmed a previous report by White *et al* (1955) who showed with both young rats and depleted adult rats that at equal levels of protein intake (6 or 9 per cent) the proteins of quinua produced gains equal or superior to those of milk protein and that quinua supplemented with milk protein did not produce better gains than quinua alone

F Nuts and Palm Kernels

Dante Costa and De Paula Fonseca (1951) carried out research on the growth promoting value of Brazil nut black beans and milk finding that the rat growth promoting value of the Brazil nut was 92 per cent of that of milk If production can be increased and the cost of the Brazil nut decreased this material could be used for child feeding alone or in combination with other protein sources

In the tropics there are other sources of vegetable proteins which with further processing could find use in preparations for the feeding of children Important among these are the palm nut oil meals such as corozo the African palm and the mbocayá palm These nut oil meals have been tested on chicks by Squibb and co-workers and their amino acid composition determined (Squibb and Wyld 1952 Squibb Aguirre and Bressani 1958) In recent years large scale plantings of the African palm (*Elais guineensis*) in Central America have made available increased quantities of palm oil meal (Reif 1951) Another palm of the genus *Acrocomia* grows in abundance from Central America to Argentina The species *Acrocomia total* mbocayá palm is of economic importance to Paraguay where the fruit is eaten by both man and animals (Bertoni 1941 Markley 1955) Their biggest disadvantage as oil meals is their large crude fibre content but it should be possible to correct this by proper processing

G Leaf Proteins

Attention has been focused for several years on the utilization of leaf protein for livestock feeding, and possibly for man Several reports in the literature indicate that leaf proteins are relatively good supplements for cereal diets (Sur and Subrahmanyan 1954 Sur 1955) Kamath and Sohonie (1956) showed that the amino acid content of leaf protein while inferior to casein is sufficiently good to be a useful potential supplement to cereal diets The amino acid composition of vegetable leaf proteins as well as the biological values of several preparations have been reported by Armstrong and Thomas (1950) Davies and Evans (1952) and Kelley and Baum (1953) The values varied from 55 to 71 per cent with very high digestibilities ranging from 76 to 93 per cent

Leaf protein preparations have been made by expressing the juice from green crops and coagulating it to obtain a product which upon drying has 30 to 60 per cent crude protein and less than 5 per cent crude fibre (Armstrong and Thomas 1950 Cowlishaw *et al* 1956*a* 1956*b*) However the results of feeding this product have thus far proved disappointing

Biological values of alfalfa protein have been found to range from 55 to 65 per cent (Armstrong and Thomas 1950 Klosterman *et al*, 1951) and both biological assays and chemical analyses have shown that methionine is a limiting factor (Klosterman *et al* 1951 Steward *et al*, 1951) Processing methods still limit the feasibility of these leaf protein concentrates

however, a successful product could be of practical importance for child feeding

1 Yeasts

Yeasts have received particular attention as protein supplements to both human and animal foods The yeast *Torulopsis utilis* which is preferred to brewer s or baker s yeast because of the greater economy of manufacture and freedom from bitterness has received special attention (Owen and Johnson 1955) Since the gross composition of the yeasts can be considerably varied by environmental conditions during produc tion strains of relatively high protein content can be produced if desired Examples of this variability in yeasts have been given by Steinberg and Ordal (1954) who studied the effect of fermentation conditions on the rate of fat production by *Rhodotorula gracilis*

1 Algae

The artificial culturing of unicellular photosynthetic organisms such as chlorella has been enthusiastically advocated by many investigators as a potential food *Nutrition Reviews* 1955 Hundley Ing and Krauss, 1956 *Nutrition Reviews* 1958) Yields of cultured algae are large and the ease with which their content of protein fat and carbohydrate can be increased or changed by relatively slight manipulations of the culture conditions could make possible the production of a variety of food types from this single source As yet very few studies have been conducted on the value of algae as the major source of protein in animal diets although the few which have been reported have indicated that up to 20 per cent of dried chlorella in the diet was beneficial for chicks and acceptable to human beings (*Nutrition Reviews* 1958 Combs, 1952)

VI PRINCIPLES OF PREVENTION

A Use of Educational Techniques

Wherever kwashiorkor occurs cultural factors such as ignorance of the nutritional needs of the child and ways of satisfying them prejudices or taboos against desirable foods and poor sanitary practices combine with economic and agricultural limitations to deter mine the prevalence of the syndrome

In preventing protein malnutrition in children educational programmes can play an important role These should involve all educational and income levels with greatest emphasis among the low socio-economic group who are the most affected Appro-priate teaching of the basic concepts of hygiene including nutrition should form part of the general educational programme and be included in campaigns against the illiteracy and ignorance which so greatly retard sound progress and economic development in technically underdeveloped areas Co-ordinated efforts need to be carried out through schools, health centres agricultural extension agencies community develop-ment projects and such other means of diffusion of knowledge and contact with local people as may be available

Basic principles of nutrition and general hygiene should be included in the curriculum of future teachers Special orientation should be given to those persons who will be teaching in rural areas These principles should be incorporated as an integral part of the general knowledge imparted by primary schools and the child inculcated with concepts that will eventually influence family and community practices

It is particularly important that social workers nurses and doctors working in health centres espe-cially in the maternal and child health programmes have adequate and practical knowledge of nutrition and apply and transmit this knowledge as part of their health education activities and other applied pro-grammes Agricultural extension workers, home economists and home demonstration agents can also make a valuable contribution to the diffusion of the concepts of good nutrition

As an example of what may be achieved when mothers are brought to a recognition of the importance of administering adequate diets to their children our observations on our own hospital treated cases of kwashiorkor may be cited When these children return to their homes after recovery and discharge from the hospital the majority continue in good nutritional condition because the mothers have been convinced, through talks prior to the discharge of the patient that a deficient diet was the principal cause of the disease Most of these mothers have proved capable of administering a more nourishing diet to their children without any alteration in the economic condi tion of the family

Unfortunately in most underdeveloped areas there is a lack of personnel trained in nutrition to fill even the minimum needs required for initiating and super vising educational programmes The training of qualified persons should therefore be a prime objec tive of organizations responsible for or interested in combating these serious nutritional problems. This must include the preparation of suitable teaching material in nutrition adapted to the local situation

Nutritional education programmes should consider existing food habits and prejudices of the population and employ to the fullest extent those sources of food which are locally available or whose production can be readily increased or developed. Very frequently personnel in charge of advising as to correct feeding

habits including physicians make the mistake of recommending foods or cooking techniques which give excellent results in other circumstances but which are impractical because they ignore local economic and cultural realities

As will be discussed separately supplementary feeding programmes should serve educational functions and they may frequently be major instruments for developing more desirable food habits

All of the above educational activities are of importance and require the co-ordinated efforts of several agencies working in an area Personnel in the field of health education agriculture and welfare should co-operate in nutrition programmes designed to prevent kwashiorkor Such co-operation however is often difficult to achieve because of the failure to realize the gravity and importance of malnutrition in areas in which kwashiorkor occurs For the best results it is essential that project leaders and professional personnel recognize the importance of the problem before undertaking popular education campaigns

B Improving Dietary Protein

1 Increased Use of Animal Protein in Mixed Diets

When diets contain predominantly protein of high biological value it is relatively easy to ingest quantities adequate to meet protein needs As protein quality drops the amount of food which must be consumed to meet protein needs increases to the point at which monotonous diets containing an unbalanced protein (e g corn or millet diets) cannot be consumed in sufficient quantities to make up for the poor quality of their protein The prevention of protein malnutrition in people consuming such foods requires an improvement in the biological value of the protein in the diet

The classical method of so doing is to increase the proportion of protein of animal origin This is the basis for various milk distribution and milk conservation programmes Increased consumption of meat eggs cheese and fish can be similarly effective Animal products are particularly good supplements to the protein of cereals so that the resulting increase in biological value has benefits beyond the protein content of the animal products themselves This is due in part to the fact that they supply adequate lysine which is a limiting amino acid in cereal proteins For example it has been demonstrated that neither wheat flour supplemented with milk and meat nor a wheat cereal product combined with 30 per cent milk powder are further improved by supplementation with lysine (Sarett 1956) When animal proteins supplement corn diets they supply important quantities of tryptophan as well as lysine (Cravioto *et al* 1955) In addition to recent amino acid studies with cereals there are of course numerous demonstrations of the improvement in protein nutrition which result from adding animal protein foods to predominantly vegetable diets (Costamaillere and Ballester 1956 Carpenter *et al* 1957 Sure *et al* 1957)

2 Complementary Combinations of Vegetable Proteins

In many areas however sufficient animal protein is not available nor likely to be in the foreseeable future Fortunately there are other means of improving dietary protein The approach which appears to have the greatest immediate practicality and on which work is most advanced is the improvement of the protein content of diets by combining protein sources of vegetable origin to improve the total amino acid pattern of the diet

Desikachar Sankaran and Subrahmanyan (1956) have demonstrated by biological trials on rats the improvement in protein value obtained by adding soya or Bengal gram to mixed local diets based on rice Phansalkar and Patwardhan (1956) have shown that the egg replacement and biological values of proteins from vegetables were higher when the protein was derived from mixtures of Indian cereals and legumes than when derived from a single cereal grain such as wheat

Several combinations of cereal grains and legumes with a high nutritive value have been described by Tongur and Orlova (1956) The best mixture tested contained 60 per cent buckwheat 20 per cent soya and 16 per cent rice and proved to be better than casein when tested in rats

Mangay Pearson and Darby (1957) showed that millet (*Setaria italica*) will correct the niacin deficiency induced in the rat by a 9 per cent casein and 40 per cent corn diet The authors also showed that the addition of 1 per cent lysine to a diet of 40 per cent corn and 40 per cent millet improved growth considerably but produced no response when added to a diet of 80 per cent corn and 10 per cent millet unless niacin and/or tryptophan were also added Millet therefore improved the tryptophan but not the lysine deficiency of corn Other studies have shown that corn and other cereal proteins can be improved biologically by adding yeast soybean flour or peanut meal (Sure 1948*a b*) Both corn and wheat flour can be improved by adding buckwheat flour as shown by Chen and Wang (1937) Orru (1940) Sure (1955) and Koyanagi Ota and Takanohashi (1956)

3 Prepared Vegetable Protein Mixtures for Child Feeding

It is clear from the preceding section that many different combinations of vegetable proteins can be

devised which are of high nutritive value and that protein quality can be improved by this means wher ever animal protein is costly or in short supply It is also quite feasible to prepare combinations of vegetable protein sources in a form suitable for the supplementary and mixed feeding of infants and young children In order to do this the following should be taken into consideration—

(a) The amino acid composition of the individual ingredients and of the final product

(b) The possible presence of toxic or interfering factors

(c) The need for obtaining exact specifications for each of the components

(d) The necessity of avoiding processes that damage the quality of the protein

(e) The desirability of using products of local origin.

(f) The fact that the final product must be inexpensive and easily preserved

(g) The requirement that it may be easily used in the home as an infant food by mothers of low income families

(h) The demand that it must not run counter to the existing dietary habits and prejudices

Promising mixtures should be analysed for their nutritive value as finally prepared They should then be submitted to careful biological testing in animals and later in children before they are recommended for general use Suitable criteria are listed in the report of the Princeton Conference on Human Protein Requirements and their Fulfilment in Practice (Waterlow and Stephen 1957)

Although the primary objective in developing such mixtures is the provision of an inexpensive supplementary source of protein whose quality is better than that in existing diets care should be taken to ensure an adequate content of the vitamins and minerals which are likely to be deficient in the local diets Otherwise the beneficial results from improved protein intake may be negated by deficiencies of other nutriments and the product fail in its purpose of improving human health Taking account of these considerations and encouraged by the success of Gómez *et al* (1952) and Dean (1953) in treating malnourished children with soya preparations, the Institute of Nutrition of Central America and Panama has developed INCAP Vegetable Mixture 8 made up of 50 per cent dried corn masa 35 per cent sesame meal, 9 per cent cottonseed press cake 3 per cent torula yeast and 3 per cent kikuyu leaf meal This mixture has proved to be a good protein source for children recovering from kwashiorkor and for the rehabilitation of under nourished children (Scrimshaw *et al* 1957*a* *b* Béhar *et al.*, 1958) A still cheaper preparation, Mixture 9 omitting the sesame oil meal is now being tested This formula which consists of 28 per cent dried corn masa 28 per cent ground sorghum, 38 per cent cottonseed press-cake 3 per cent torula yeast and 3 per cent kikuyu leaf meal is scheduled for field use in Central America in the near future

The Indian Council of Medical Research (Subrahmanyan Patwardhan and Moorjani 1955) has reviewed work carried out in India and China on the preparation testing and use of milk substitutes for child feeding in areas where milk production is inadequate and concluded that simple preparations from soybean, peanuts, cashew nuts coconut, or legumes can be helpful in supplementing poor cereal diets These workers have also described the development, testing and economic aspects of a multipurpose food composed of 25 per cent Bengal gram grits and 75 per cent peanut cake grits fortified with calcium phosphate thiamine riboflavin and vitamins A and D in suitable proportions The multipurpose food was tested as a supplement to poor cereal diets for under nourished children and in the treatment of nutritional oedema and found effective

Senecal (1958) has reported a mixture of millet and peanut press-cake to give good results in the treatment of kwashiorkor A vegetable milk prepared from peanuts has been tested by Holemans Lambrechts and Martin (1956) and found to be a good supplement as judged by both biochemical analyses and clinical indices. Hansen (1958) has had good success in treating kwashiorkor and in demonstrating satisfactory nitrogen retention in children using a mixture containing 33 per cent whole ground corn (mealie meal) 33 per cent corn germ and 33 per cent green cowpea flour (*Pisum arvense*)

4 Addition of Synthetic Amino Acids to Vegetable Diets

Improvement of poor-quality protein or of amino acid deficiencies in food protein can also be achieved by the addition of the deficient amino acids. In under developed areas the amount of animal protein consumed is low and there is a corresponding increase in consumption of cereal grains, which contain protein deficient in several amino acids For this reason Flodin (1953 1956) concludes that cereal grains are the favourable vehicles for amino acid supplementation

Scrimshaw *et al* (1958) and Bressani *et al* (1958) have shown that a significant improvement in nitrogen retention can be obtained upon addition of tryptophan, lysine and isoleucine to corn masa flour Mosqueda-Suarez (1955) found that the addition of 0 2 per cent tryptophan to a de germed corn product arepa , increased the rate of weight gain in rats. The addition of lysine alone also increased weight

gain but not as much as that obtained with tryptophan with the addition of both amino acids the rate of increase in growth was highly significant

Several recent studies (Westerman Kannarr and Rohrbough 1957 Westerman Hays and Schoneweis 1957) have once again demonstrated the finding of Osborne and Mendel (1914) that the addition of lysine to wheat products results in an improvement in their protein quality That the addition of lysine not only improves the growth rate of rats but also prevents the development of fatty livers where cereal diets are fed at a 5 4 per cent protein level has been shown by Deshpande Harper and Elvehjem (1957)

Other vegetable proteins have also been successfully supplemented with synthetic amino acids Demonstrations of the beneficial effect of supplementing most animal feeds with methionine and lysine have been recently reviewed by Rosenberg (1957) Cowlishaw *et al* (1956*a* *b*) have shown that concentrates from the proteins of leaves as feed for chicks are improved by the addition of lysine but not by methionine Kik (1956*a* *b*) demonstrated that the proteins of rice and rice products are improved by the addition of 0 2 per cent lysine and 0 2 per cent DL threonine and that the addition of 0 5 per cent DL methionine 0 5 per cent DL threonine and 0 4 per cent L lysine to barley resulted in a significant increase in the protein efficiency ratio The same author has also shown that supplementation with 0 5 per cent each of DL threonine and DL methionine improved peanut flour as measured by weight gain in rats and protein efficiency ratio

Examples of the experimental evidence for the biological effects of amino acid supplementation are cited because of their promise for the future At the present time however knowledge of amino acid requirements and interrelationships are incomplete and the costs of synthetic amino acids other than methionine and possibly lysine too high to make amino acid supplementation a practical procedure for improving protein quality of human diets

5 Selection of More Nutritive Varieties of Basic Food Crops

Another method of improving vegetable proteins deserves mention Improved varieties of some basic food crops of higher nutritive value can be developed by selective breeding Some of the factors affecting the amino acid composition of soybean have been studied by Krober (1956) who reported significant differences in methionine concentration among fourteen varieties of soybeans The author concluded that it is practical through selection to increase the methionine content of soybean The factors influencing the methionine lysine and tryptophan content of twenty five varieties of beans (*Phaseolus vulgaris*) were studied by Tandon *et al* (1957) who reported that the overall differences in nitrogen and tryptophan content among varieties and between localities were highly significant This means that through selection of varieties for localities nutritionally better beans could be developed Other studies on beans which have appeared recently also show the effects of environmental and genetic factors on the nutritive value and suggest the possibilities for breeding more nutritious varieties (Lantz, Gough and Campbell 1958)

Cereal grains are in general the most important staple foods in underdeveloped areas (F A O 1956, Sebrell and Hand 1957) and for this reason more attention should be given to possibilities of improving their protein quality Corn is such an important staple food in many parts of the world that numerous studies have been directed toward its biological value Hogan *et al* (1955) have shown that although the biological value of high protein corn (16 1 per cent) was inferior to that of the low protein corn (7 3 per cent) the former was superior per unit weight in its ability to satisfy protein requirements Similarly Reussner and Thiessen (1957) have shown that two high-oil protein corns fed *ad libitum* to rats give better growth and protein efficiency than ordinary corn even though inferior on an equal nitrogen basis Similar results have been reported by other investigators in pigs (Dobbins *et al* 1950) rats and chicks (Mitchell Hamilton and Beadles 1952 Sauberlich Chang and Salmon 1953*a*) The amino acid composition of corn and factors affecting it have also been extensively studied and the results in general confirm the possibilities of selecting more nutritious varieties (Miller Aurand and Flach 1950 Sauberlich Chang and Salmon 1953*b* Wolfe and Fowden 1957 Bressani and Mertz, 1958) Other cereal grains have also been investigated so as to find out their deficiencies in order to improve them by genetic means and for the better complementation with other food proteins (Frey Hall and Shekleton 1955 Baptist and Perera 1956 Weber *et al* 1957, Gunthardt and McGinnis 1957 Adrian and Sayerse 1957)

6 Improvement of Protein Quality of Foods by Processing

Finally improvement in nutritive value of their protein can result from cooking roasting or other processing of foods An interesting and classical example is the lime treatment which corn for human consumption receives in the Latin American countries (Bressani and Scrimshaw 1958) Several investigators have shown that this treatment gives a product of better nutritional qualities than the raw material as judged by rat and pig growth studies (Squibb *et al* 1955 Kodicek *et al*, 1956 Pearson *et al* 1957) Beans and other legume seeds also gain in nutritive

value from cooking or roasting (N R C 1950 Siener 1950 Carpenter, 1958) Care should be taken how ever to determine the optimum treatment conditions to obtain a product with the desired characteristics over treatment may have serious adverse effects on nutritive value

C Instituting Supplementary Feeding Programmes

The permanent correction of nutritional deficiencies in a population requires measures leading to improved food production education and public health These are inherently difficult and long range measures while the direct distribution of supplementary food to vulnerable groups is a method of combating malnutrition which is in theory capable of immediate application It has been widely recommended and frequently attempted as both an emergency and a transitional measure for getting more and better food to those in greatest need School feeding programmes have also been adopted on a semi permanent basis in many of the more highly developed countries as part of educational activities in nutrition

Unfortunately while supplementary feeding programmes are well adapted to the nutritional improvement of individuals in captive populations as in school and public institutions frequently the bulk of persons in nutritional need in an underdeveloped country cannot be reached through existing public channels For example the prevention of kwashiorkor requires that children shall receive supplements of good quality protein long before they reach school age since the requirements for protein per unit of body weight are high and the customary diets shall be least adequate in this age group Supplementary feeding programmes for pre school children can be effectively organized only with a great deal of effort and the use of relatively numerous and well trained auxiliary personnel Although great need exists in rural areas, they are least practical where the population is scattered

Nursing mothers and to a lesser extent women during pregnancy also merit special protection from nutritional deficiency because of their higher requirements during these physiological periods Attention to them is also justified on the basis of the possible adverse influences of nutritional deficiency in the mother on the health of the child To the extent to which countries have the human and material resources to organize effective programmes for the distribution of food to these vulnerable groups, the assistance of U N I C E F and other governmental and non governmental agencies in helping to make available suitable food for supplementary feeding programmes, at little or no cost is of incalculable value

Needless to say such programmes should provide the necessary elements for a good diet and to be safe and effective should not overlook any of the major deficiencies Fortunately the dried skimmed milk widely available for this purpose provides protein of excellent quality in a concentrated and inexpensive form as well as abundant riboflavin and calcium. Thus with the exception of vitamin A it supplies the nutriments most lacking in many areas Although skimmed milk is lacking in fats and fat soluble vitamins its use presents no problem as long as attention is given to supplying a supplementary source of vitamin A when this nutriment is found to be critically low in the basal diet

U N I C E F has given invaluable world wide support to supplementary feeding programmes in areas of nutritional need particularly through the distribution of powdered milk and where needed vitamin A concentrates A primary objective of these programmes has been to stimulate the local consumption of milk and then to co-operate with F.A O in assisting countries to improve milk production and conservation Particularly good progress has been achieved in countries where powdered milk plants have been successfully established and a marked increase in the production and utilization of milk and milk products observed as a consequence In areas in which these efforts alone will not suffice to ensure adequate protein supplies for infant and child feeding similar programmes for the production and distribution of suitable vegetable mixtures are contemplated

Thus far little has been said concerning the use of supplementary feeding programmes for improving the nutrition of school children Although there are some areas in which this has importance as a health measure in most regions correction of nutritional deficiencies through supplementary feeding would require concentration on the groups previously mentioned—pre school children lactating mothers and pregnant women The more important opportunity presented by school feeding programmes is an educational one Children can be taught to eat a greater variety of foods and the school snack or meal can be made the basis of a nutrition education programme involving parents as well as children. To neglect the educational aspects of the school feeding programmes as is so widely the case at present is a serious mistake

The development of school gardens and small animal raising projects in connection with school feeding programmes offers a further opportunity to provide instruction in nutritional principles as well as permitting the introduction of measures to improve the agricultural production of protective foods

D Development of Nutritional Rehabilitation Centres

Much more frequent than frank kwashiorkor is the border line or sub-clinical case of protein malnutrition with a varying degree of growth retardation, apathy

mild skin and mucous membrane lesions muscular wasting weakness and slight oedema The term pre kwashiorkor has been used to describe this condition which characterizes large numbers of pre-school children in areas in which kwashiorkor occurs

Generally when seen in clinics and health centres these pre kwashiorkor children are not considered ill enough to merit hospitalization and it is customary to attempt to treat them as out patient cases especially when hospital services are already overcrowded with more severe cases In these circumstances the ordinary treatment too often consists of a hurried prescription for a proprietary product or a general admonition to feed the child better without considering either the economic limitations of the family or the failure of the mother to understand the instructions Lack of time interest or full comprehension of the problem on the part of the physician or nurse often results in failure to emphasize proper feeding and how it may be attained Even when a recommended dietary supplement is purchased the mother may be unprepared to overcome the child s anorexia or resistance to a new food The child thus inexorably develops the severe form of the disease and eventually requires costly hospitalization or else may die in the meantime of an intercurrent infection The final cost in loss of life or prolonged hospitalization is high

In order to care for these children the establishment of recovery centres has been suggested These are low-cost day-care centres requiring less personnel and expense than hospital facilities and organized to meet local conditions and needs where the children can receive adequate food throughout the day to improve their nutritional status The centres may give the best results if some participation in the preparation and administration of food can be arranged for the mothers Such institutions can also make possible earlier discharge of hospitalized children by accepting them in the recovery phase when the only further treatment needed is an adequate diet

The recovery centres can perform a very important function in the prevention of kwashiorkor through both the direct improvement of the child s diet and the education of the mother They can also greatly help the hospitals by reducing the number of cases that reach the stage of requiring hospitalization and by permitting an earlier dismissal of the treated cases without the danger of relapse Obviously the care of children in such a centre after a period of hospitalization for the treatment of kwashiorkor is not only advisable for them but it is also far less expensive

E Environmental Sanitation Procedures

There is increasing recognition of the extent to which diarrhoea of infectious origin and other infectious diseases act synergistically with poor dietary habits to result in kwashiorkor and other nutritional deficiencies In addition episodes of dysentery and other infectious diseases may prove fatal in malnourished subjects while they would not have serious consequences for well nourished individuals

The high frequency with which infections appear in the recent medical histories of children hospitalized with kwashiorkor has already been mentioned Not only do the majority of Guatemalan children hospitalized for kwashiorkor for example give a history of diarrhoea with fever but other illnesses such as measles and whooping-cough sometimes appear to precipitate this syndrome Unfortunately environmental sanitation is likely to be poor in precisely those areas in which feeding practices for young children are grossly inadequate In rural Guatemalan towns where kwashiorkor is a major cause of death almost 8 per cent of all children under ten years reveal Shigella organisms following the culture of a single rectal swab from each child (Beck Muñoz and Scrimshaw 1957) Since a single examination detects only part of the infection present true Shigella prevalence among these children may be somewhat higher There is also a possibility that other micro-organisms not normally pathogenic may become so when the child is grossly debilitated by malnutrition

In areas in which kwashiorkor is of public health importance intestinal parasites are usually very common and constitute a further burden on the malnourished child Although there is some direct evidence that moderate to severe infestation with *Ascaris lumbricoides* interferes with the digestion and absorption of protein (Venkatachalam and Patwardhan 1953) we feel that the role of intestinal parasites in the development of the syndrome has sometimes been overestimated and overstated by professional as well as lay persons

There is a general tendency to attribute the majority of deaths among young children to Ascaris and other intestinal parasites instead of to the malnutrition diarrhoea and other infectious diseases which are primarily responsible for the high mortality in children in underdeveloped areas In Central America this has the further harmful effect of encouraging the giving of drastic purgatives to rid the child of worms with a resulting aggravation of the diarrhoeal disease and the already poor nutritional status Unfortunately the efficacy of this treatment is demonstrated to lay minds by the Ascaris commonly excreted in diarrhoeal stools and expelled in still larger numbers when purgatives and vermifuges are given

It should be abundantly clear that in most technically underdeveloped areas the problems of nutrition and infection are closely related particularly for young children Progress in reducing enteric infection and

infestation through the improvement of water supplies and disposal of excreta and by the introduction of other sanitary measures will help to decrease deaths from malnutrition just as improving nutrition will lower mortality from infectious diarrhoea

While improved environmental sanitation will not correct the underlying protein malnutrition which limits the growth and maturation of very large numbers of children in underdeveloped areas it will allow more of them to survive the critical pre school years If they live to school age cultural factors will usually result in an improvement in their diets In addition, sanitary measures may contribute to the improvement of the efficiency and working capacity of the parents and thus enable the family to improve their nutritional status

VII MAGNITUDE OF THE PROBLEM

Published studies and the experience of health workers in most of the technically underdeveloped countries indicate that child deaths from malnutrition including kwashiorkor are exceedingly common Owing to the way in which the *International Classification of Diseases, Injuries and Causes of Death* is compiled, however the official vital statistics of the country and the cumulative reports published by W H O fail to reveal the magnitude of the problem Furthermore the principal sign of underlying protein malnutrition in a population slowing of growth and maturation is regarded as normal for these areas and indeed often described as a racial characteristic Finally for children dying of an intercurrent infection which would not have been fatal in a well nourished child there is no record or even good means of evaluating the role of malnutrition in the death

As a consequence the magnitude of the protein malnutrition problem from the standpoint of both morbidity and mortality is usually grossly underestimated This leads to a disproportionate investment of time and money on other more obvious or better reported problems The matter is not a simple one since infections of all kinds particularly enteric are major determinants of the final appearance of clinical kwashiorkor In these circumstances programmes for the improvement of environmental sanitation have an added significance It should be evident however that no amount of infectious disease control or eradication will eliminate protein malnutrition in the young child except as it indirectly results in an improvement in the productivity and economic well being of the population The elimination of protein malnutrition in the young child requires extensive health education efforts and the availability of cheap and effective protein rich foods

The difficulties in interpreting the magnitude of the problem from vital statistics are well illustrated by an investigation of the causes of death in children under fifteen years of age in four rural communities in the highland area of Guatemala (Béhar Ascoli and Scrimshaw 1958) This study revealed that the death rate was 19 8 per 1 000 population in these communities and for children of one to four years of age 50 3 The infant death rate was 136 8 per 1 000 live births. As determined by questioning the parents, usually within forty-eight hours after the death occurred of the 222 deaths 49 were attributed to *Congenital Malformations and Diseases Peculiar to Early Infancy*" 42 to *Diseases of the Respiratory System* 37 to *Diseases of the Digestive System* (principally infectious diarrhoea) 27 to *Infective and Parasitic Diseases* 43 to *Other Specified Causes* (almost all of these kwashiorkor) 17 to *Ill Defined or Unknown*" while 7 could not be investigated The important point is that in contrast to our classification given above only one of these deaths the only case in the entire series that died in a hospital and was medically certified was classified as due to nutritional deficiency in the official statistics while 58 were listed officially as dying from intestinal parasites— worms " 'worm attack worm fever etc —and even though Ascaris infestation in the Guatemalan highland is widespread, it is impossible to conceive that Ascaris *per se* are responsible for these many deaths

Persons concerned with preventive programmes in areas in which hospital cases and knowledge of the population make it evident that kwashiorkor is a serious public health problem will doubtless find it desirable to make similar studies in their own areas. Such studies are useful for the proper interpretation of the official vital statistics and helpful in focusing the attention of health and other government authorities on the magnitude of the problem. Studies of differences in the growth and maturation among different socio-economic groups and of morbidity from diarrhoeal disease are also likely to prove revealing and helpful in defining the problem.

VIII ROLE OF GOVERNMENTAL AND NON-GOVERNMENTAL AGENCIES

It should be apparent from the discussion of factors responsible for the development of clinical cases of kwashiorkor that the task of its prevention within a country cannot be left to specialized nutrition personnel nor to any single programme or agency Governmental departments, whose job is to work

with people and non governmental welfare agencies should be made to understand the magnitude of the problem and should contribute to its solution

It is obvious that agricultural agencies will help by working toward a greater availability of animal and plant sources of protein for human consumption Health agencies will assist both by disseminating information as to desirable feeding practices for young children—through health education maternal and child health, and health centre programmes—and also through programmes to improve environmental sanitation and reduce the spread of infectious disease The training of schoolteachers to include principles of nutrition in the curriculum as well as in their programmes with parents is also an important part of a determined national effort to abolish kwashiorkor

Supplementary feeding programmes are usually maintained by a variety of agencies of both governmental and private nature within a country To the extent to which these programmes can reach preschool children and nursing mothers they will contribute to reducing the magnitude of the problem The value of rehabilitation centres should also be recognized and established as a vital link between outpatient and hospital care of malnutrition cases The hospitals can do a great deal by educating the parents of their kwashiorkor patients as to the cause and cure of the disease in a way that will help to avoid recurrences and benefit other children in the family

The health department should maintain liaison with the economic and industrial development plans of a country to be sure that these do not conflict with health objectives For example governments should not encourage the production of cassava flour in a country where cassava is not now in general use for it is almost certain that the product will be used for infant feeding with unfortunate consequences In some countries the leadership of a national nutrition council integrated by representatives from all of the governmental and perhaps from the major non-governmental agencies concerned with matters directly influencing nutrition policy or administering programmes relating to the improvement of the nutritional status of the population may be desirable In others this leadership may well be provided by the Nutrition Section of the Health Department or by a National Institute of Nutrition The prevention of kwashiorkor is such an enormous job that without comprehension of the problem and co-operation at all levels progress will be slower than would otherwise be the case and thousands of additional children will die needlessly from kwashiorkor

IX. CONTRIBUTION OF INTERNATIONAL AGENCIES AND PROGRAMMES

Just as the national ministries and agencies must co-ordinate their efforts to combat malnutrition and to eliminate kwashiorkor so should the International Agencies co-operate in the common effort and encourage national elements to do so While countries will naturally look to W H O for guidance in the medical aspects of kwashiorkor supplementary feeding programmes for this vulnerable group and the improvement of environmental sanitation F A O should be prepared to give technical assistance in problems relating to food production and surveys of the dietary habits of population Both agencies together with U N E S C O are likely to be involved in educational efforts and U N I C E F has been and will continue to be a major resource for the provision of food supplements in areas in which protein is inadequate as well as for supplies and materials for the combat of infectious diseases

The problem of preventing kwashiorkor and other forms of protein malnutrition is so large and important that each of the specialized agencies of the United Nations mentioned has a responsibility in this area It is to be hoped that the future will see a growing realization of these obligations and of their material and human resources to help meet them Valuable assistance can also be rendered by the various bilateral technical assistance programmes between countries

X SUMMARY

The underlying cause of kwashiorkor is a relatively severe deficiency of protein and treatment consists primarily in giving the child a diet rich in protein of good quality and as soon as initial anorexia is over come ensuring that the diet contains all the other essential nutriments in physiological amounts Since children have often suffered considerable diarrhoea before admission it is frequently necessary to give special attention to restoring electrolyte balance and it is best to give penicillin until the acute danger of broncho pneumonia is past

The disease occurs where diets of young children fail to contain sufficient protein especially in the months following weaning and is therefore highly susceptible to stress factors The most important of these diarrhoea of infectious origin is exceedingly common in most areas where kwashiorkor is endemic, and precedes the development of clinical signs in a

large proportion of cases Other stress factors are the common contagious diseases

Technically underdeveloped areas in general do have adequate potential protein sources which have not been exploited Where animal protein production is inadequate mixtures of vegetable proteins, with a high biological value can be prepared for the supplementary and mixed feeding of infants and young children To a considerable extent the problem is also one of ignorance and lack of proper feeding habits rather than inability of the parents to supply protein to the child

The prevention of kwashiorkor requires understanding of the problem on the part of officials and other personnel in the fields of health education and agriculture as well as their active co-operation in the development and application of prophylactic measures Similarly the technical agencies of the United Nations W H O, F.A O., U N I C E.F and U N E S C O can be of major assistance to countries in various aspects of the problem

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5

Basal Metabolism in the Japanese Population

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Contents

I INTRODUCTION

This paper deals with the results obtained from a number (more than 2,500) of basal metabolism measurements conducted in our laboratory since 1950

Before proceeding it is necessary to mention the conventional BM measurement conditions previously accepted In our BM measurements all conditions except room temperature were imposed upon all subjects who willingly obeyed the instructions

Although it is considered most desirable to maintain room temperature at 20–25 C, in a country like Japan where home heating conditions are inadequate room temperature is controlled by external temperature Room temperature in winter is in most cases below 20 C usually around 10 C Our observations carried out in winter were therefore mainly conducted at room temperatures around 10° C

Despite the above some Japanese workers have raised objection to our observations conducted around 10° C on the ground that it is impossible to estimate BM at such a low temperature

We however do not agree with this point of view because we consider that measurement conditions are satisfactory even in a low temperature in the vicinity of 10° C provided the subject is in a warmly clothed comfortable condition since we have obtained results which support our view

In recent years objections of this kind have hardly ever been raised

The key factor affecting BM estimation is not room temperature but the under-clothing climate (climate between skin surface and clothing) because it is only at an extremely low room temperature far below 0 C that one observes the rise of metabolism caused by cold stress which arises only from the exposed skin surface

Thus the maintenance of room temperature above 20 C in BM measurement is not considered to be an essential factor in such observations

II CLIMATE AND THERMAL CONDITIONS

A Seasonal Variation

It may be said that there is a divergence of opinion regarding seasonal variation in BM some workers agree on the existence of such a variation while others reject this theory

Our results indicate that the BM for each month of the year is in exact inverse proportion to the average external temperature of each season (Suzuki 1957*a*) The following table (Table 5 1) gives the BM values of five subjects (laboratory research workers) which were observed twice a month for a period of 3–8 years Fig 5 1 illustrates the correlation between the

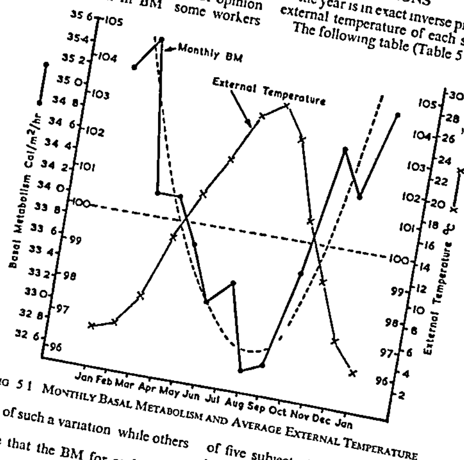


FIG 5 1 MONTHLY BASAL METABOLISM AND AVERAGE EXTERNAL TEMPERATURE

TABLE 5 1

Monthly Basal Metabolism

Subject	Date	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Avrge
Undernutrition	1949	—	—	—	—	—	—	—	29 6	—	30 1	32 8	32 7	31 3
	1950	34 1	35 2	32 8	—	32 3	—	33 1	29 8	30 9	34 0	—	36 2	33 2
	1951	36 6	37 1	36 5	—	36 5	31 4	29 6	33 9	32 0	32 0	32 5	34 0	33 7
S S ♂	1952	34 3	34 3	33 8	34 0	33 4	32 2	34 2	32 4	32 6	33 0	32 8	—	33 4
	1953	35 1	35 7	—	36 2	33 9	32 3	31 5	33 8	34 2	33 2	36 2	35 6	34 3
	1954	34 0	36 2	34 3	34 6	33 3	33 3	33 8	31 9	31 8	32 9	34 4	33 7	33 7
	1955	35 3	34 2	31 9	32 0	31 3	31 7	32 7	32 5	31 7	33 7	34 6	33 2	32 9
	1956	33 7	34 5	32 4	—	30 7	34 4	31 1	30 2	31 0	30 6	31 5	32 8	32 1
44 yr →	1957	31 1	32 0	32 5	32 3	30 8	31 7	30 0	30 7	—	31 1	31 1	34 1	31 6
1952–57 →	Average	33 9	34 5	33 0	33 8	32 2	32 6	32 2	31 9	31 3	32 4	33 4	33 2	32 9
	%	103	105	100	103	98	99	98	97	95	98	102	101	100
Undernutrition	1949	—	—	—	—	—	—	—	30 1	—	29 6	31 8	32 1	30 9
	1950	31 2	31 6	32 7	—	32 1	—	31 5	28 7	31 6	34 5	—	36 8	32 3
	1951	37 2	35 6	32 9	—	—	32 0	31 4	32 4	31 5	34 2	32 2	32 6	33 2
S N ♂	1952	32 2	34 3	33 2	32 4	32 1	32 1	32 2	33 4	32 3	34 4	34 7	—	33 0
	1953	36 4	35 9	—	34 5	32 1	31 0	32 3	32 7	33 2	34 5	33 8	34 4	33 7
	1954	35 4	36 3	32 7	31 6	33 8	32 8	33 7	32 7	32 8	33 6	34 3	33 8	33 6
	1955	33 2	32 2	33 7	34 7	34 0	31 9	34 2	32 7	33 6	33 3	34 4	33 5	33 5
	1956	33 1	34 6	35 3	33 1	33 9	32 6	33 6	33 3	32 0	31 7	31 6	33 5	33 2
41 yr →	1957	34 8	32 4	34 5	33 4	—	33 6	33 2	31 7	—	32 3	34 1	34 0	33 4
1952–57 →	Average	34 2	34 3	33 9	33 3	33 2	32 3	33 2	32 7	32 8	33 0	33 8	33 8	33 4
	%	102	103	101	100	99	97	99	98	98	99	101	101	100
S K ♂	1954	—	—	33 0	33 8	33 4	32 6	33 4	31 8	32 3	35 0	34 3	36 4	33 6
	1955	36 9	34 3	34 1	34 7	34 3	34 9	33 2	33 1	33 0	33 7	34 8	34 3	34 3
	1956	36 6	36 5	33 9	32 6	—	32 0	31 3	31 4	32 2	33 0	34 8	34 0	33 5
45 yr →	1957	33 4	32 8	32 4	33 2	—	—	—	—	—	—	—	—	32 9
	Average	35 6	34 5	33 4	33 6	33 9	33 2	32 6	32 1	32 5	33 9	34 6	34 9	33 7
	°	106	102	99	100	101	98	97	95	96	101	103	104	100
T K ♂	1954	—	—	34 7	35 9	35 2	34 3	35 4	34 2	34 5	36 6	35 6	35 5	35 2
	1955	35 9	39 0	35 1	36 8	34 6	34 4	34 7	34 4	35 6	35 0	38 0	37 7	35 9
	1956	36 5	37 4	36 7	34 7	36 0	34 7	35 4	33 9	32 2	—	37 9	34 3	35 4
28 yr →	1957	—	34 7	37 2	34 3	34 4	35 6	35 5	34 4	—	34 6	34 1	34 0	34 9
	Average	36 2	37 0	35 9	35 4	35 1	34 8	35 2	34 2	34 1	35 4	36 4	35 4	35 4
	%	102	105	101	100	99	98	99	97	96	100	103	100	100
S O ♀	1954	—	—	35 4	35 0	34 9	34 4	35 3	33 1	34 3	34 3	36 7	36 1	35 0
	1955	35 6	35 1	35 8	34 1	32 7	32 7	32 5	33 8	32 7	33 7	36 1	33 7	34 0
	1956	37 1	39 3	34 7	34 6	34 1	32 4	32 5	32 2	30 8	33 0	36 3	36 3	34 4
26 yr →	1957	36 1	36 8	33 2	33 4	34 9	32 9	34 6	30 1	—	31 4	33 1	33 0	33 6
	Average	36 3	37 1	34 3	34 3	34 2	33 1	33 7	32 3	32 6	33 1	35 6	34 8	34 3
	%	106	108	100	100	100	97	98	94	95	97	104	101	100
Average of all		35 2	35 5	34 1	34 1	33 7	33 2	33 4	32 6	32 7	33 6	34 8	34 4	33 9
°		104	105	101	101	99	98	98	96	96	99	102	101	100
Monthly average external temperature		3 2	3 9	7 0	12 8	17 2	20 8	25 1	26 4	22 6	16 4	11 0	5 7	14 3
Absolute temperature °		96	96	97	100	101	102	104	104	103	101	99	97	100
Monthly average relative humidity		62	62	64	70	75	79	80	80	80	78	72	67	72

total of the above average monthly BM values and the average external temperature of each month

From Table 5 1 and Fig 5 1 we can say that BM levels show an inverse proportional relationship to the average external temperature This is further verified on investigating the quantitative relationship between the percentage BM fluctuation and the average external temperature it is found from the readings of the interior scales (percentages on temperature scale are calculated on absolute temperature) on both axes that the two are in complete agreement

observed in April and July may be explained by the light clothing during the seasonal change between spring and summer while the change to heavy clothing may be the cause of the fall in December

B Provincial Difference

In a country which is long and narrow compared with its area such as Japan there is a considerable provincial difference in external temperature between the northern and the southern regions It would therefore naturally be expected that the

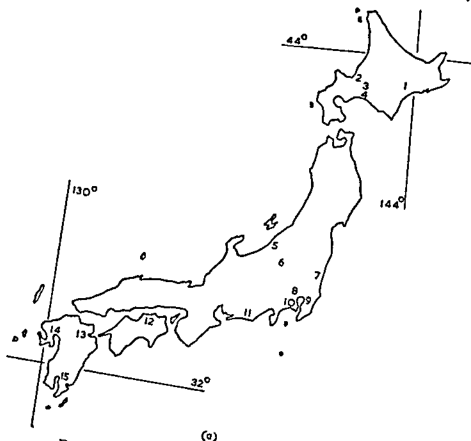


Fig 5 2 (a) Locations of Subjects Measured

This quantitative relationship between BM and external temperature is very interesting though it is based on present Japanese daily life conditions and may be inapplicable to people of other nations

From these data the seasonal BM variation of the Japanese race is quantitatively estimated as—

$\frac{1}{273}$ for a variation of 1° C that is slightly lower than 4 per cent for a variation of 10° C

Furthermore if there is significance in the rise and fall of seasonal variation BM curves the slight rise

external temperature difference would have some effect on the BM of the population

Though we have already mentioned that seasonal BM shows a close relationship to external temperature there still remains room to doubt whether an exactly identical relationship to regional climate difference can exist because in cold and warm regions clothing and heating *i e* artificial climatic conditions differ besides this the difference of the time during which subjects are exposed to such artificial temperature must also be considered

Taking winter for example heating conditions are satisfactory in the northern region and people spend more of their every-day life indoors while in the southern region though the external temperature is not excessively low room temperature is almost equal to external temperature as heating conditions are unsatisfactory and people are under the influence of the external temperature all day long

From this point of view we have carried out BM measurements on personnel of the Self Defence Forces since 1950, *a total of 1 453 persons selected throughout* the country mainly in summer and winter (Kawada, 1953 Hattori *et al* 1954*a b c*, Sujuki *et al* 1954*a b, c* Suzuki 1957*b*)

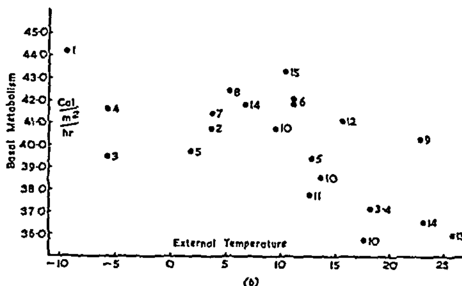


Fig 5 2 (*b*) External Temperature and Basal Metabolism of All Subjects

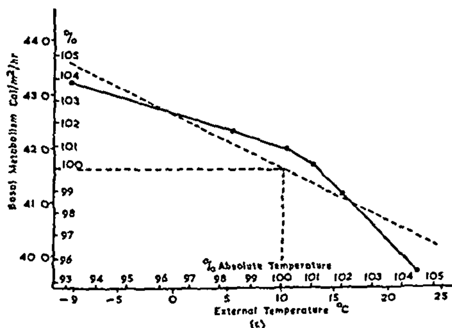


Fig 5.2 (*c*) External Temperature and Basal Metabolism

These men were selected as subjects because they are scattered all over the country (Fig 5.2a) and also because their labour and other living conditions are identical

Results of BM measurements are presented in Figs 5 2*b* and 5 2*c*

From these two figures the following tendencies are evident—

(1) BM values are in inverse proportion to external temperature

(2) BM increases sharply with temperature decrease down to 10 C. At temperatures above 10 C an increase of approximately 4 per cent for each 10 decrease of temperature is observed the coefficient being almost in consistence with seasonal variation

(3) At temperatures lower than 10 C the slope of the curve decreases and there is a 1–2 per cent increase in BM with a 10 decrease in temperature

The trend referred to in (2) and (3) is the exact reverse of that observed in naked cold-exposure in an air conditioned room As already mentioned this may be attributed to protection from cold by means of heating and clothing and spending more of the daily life indoors

Over the entire temperature range there is an average increase in BM value of approximately 2 5 per cent for a 10 C decrease in temperature

C. Calorie Intake

As already described the influence of thermal conditions upon BM has been clarified and the same tendency might be expected in the calorie intake per day

On the other hand as there are more complicated and diverse factors influencing the calorie intake than those involved in BM calorie intake fluctuation may not always be correlated with BM fluctuation

Taking such a view we investigated the relation between calorie intake fluctuation and BM fluctuation by rearranging the data of the National Nutrition Survey conducted by the Ministry of Welfare and Health

The calorie intake per adult per day of about 15 000 persons residing in thirty four cities in Japan was observed in May August and November in 1952 and in February 1953 mean monthly external temperatures in these cities were also observed (Kawada 1954*a* Kawada and Kuga 1954*b*)

From the results shown in Table 5 2 we were able to find the following tendency—in regions having a lower external temperature the calorie intake is increased while it is decreased in regions having a higher external temperature

This tendency is also indicated by the distribution of the dots in Fig 5 3

It was observed that the actual calorie intake

TABLE 5.2

Average of Calorie Intake and Percentage of Increase or Decrease in Calorie Intake for the Difference in Temperature at Various Cities in Japan

Cities	May 1952 Groups	Families	Persons	Converted ratio for man	Cal man day (A)	σ	August 1952 Groups	Families	Persons	Converted ratio for man	Cal/man day (B)	σ	Difference: Diff in temp (C)	σ	$\left(\frac{(B)}{(A)}\times 100-100\right)$ / for (C)	$\left(\frac{(B)}{(A)}\times 100-100\right)$ / for 10 C
					Cal	Cal					Cal	Cal	C	C	/ C	/ C
Tokyo	12	6 7	311[illegible]	0 825	2395	±132 3	12	608	2933	0 84	2281	±312 5	8 7 ↑	±0 00	↓ 4 8 8 7 ↑	↓ 5 5/10 ↑
6 Large Cities	26	1 14	5699	0 831	[illegible]	±131 8	26	1187	5510	0 840	[illegible]340	±232 1	9 2 ↑	±0 53	↓ 3 4/9 2 ↑	↓ 3 7/10 ↑
12 Medium-sized Cities	20	1076	5[illegible]57	0 825	2406	±124 4	20	1054	5097	0 835	2359	±214 2	9 3 ↑	±0 58	↓ 3 0/9 3 ↑	↓ 2 2/10 ↑
16 Small Cities	16	1060	5000	0 852	[illegible]445	±184 2	16	1057	4931	0 844	2363	±174 7	9 3 ↑	±0 52	↓ 3 3/9 3 ↑	↓ 3 5/10 ↑
All Cities (34)	62	3350	15956	0 834	24 3	±152 2	62	3298	15538	0 839	2349	±207 1	9 3 ↑	±0 54	↓ 3 0 9 3 ↑	↓ 3 2/10 ↑

Cities	Nov 1952 Groups	Families	Persons	Converted ratio for man	Cal man day (D)	σ	Feb 1953 Groups	Families	Persons	Converted ratio for man	Cal man/day (E)	σ	Difference: Diff in temp (F)	σ	$\left(\frac{(E)}{(D)}\times 100-100\right)$ / for (F)	$\left(\frac{(E)}{(D)}\times 100-100\right)$ / for 10 C
					Cal	Cal					Cal	Cal	C	Cal	/ C	/ C
Tokyo	24	613	2837	0 835	2304	±149 5	24	60[illegible]	2980	0 833	2370	±100 2	7 7 ↓	±0 00	↑ 2 8/7 7 ↓	↑ 3 6/10 ↓
6 Large Cities	50	1154	5247	0 837	2396	±189 6	51	1165	5412	0 839	2475	±221 9	7 9 ↓	±0 70	↑ 3 3/7 9 ↓	↑ 4 2/10 ↓
12 Medium-sized Cities	38	973	4772	0 844	2491	±242 3	40	1009	4989	0 833	2530	±250 9	8 4 ↓	±0 54	↑ 1 6 8 4 ↓	↑ 1 9/10 ↓
16 Small Cities	40	10 6	4840	0 847	2461	±275 5	40	1016	4731	0 844	2513	±217 4	8 5 ↓	±0 58	↑ 2 1/8 5 ↓	↑ 2 5/10 ↓
All Cities (34)	128	3153	14859	0 843	2444	±238 9	131	3190	15132	0 839	2503	±247 2	8 2 ↓	±0 67	↑ 2 4/8 2 ↓	↑ 2 9/10 ↓

↑ means increase in temperature or calorie intake ↓ means decrease in temperature or calorie intake

During the course of this investigation the following experiment already mentioned in the introduction was also carried out to determine the effect on BM of cold exposure in an air-conditioned chamber exposure being restricted to the face surface in this case (Suzuki *et al* 1956c)

The subject was first placed in a recumbent position *with his head through an oval wall-opening* (30 × 45 cm) connecting a cold chamber with an adjoining hot chamber so that he was lying face upward with his face only exposed in the cold room the temperature of which was 0° C or −16° C, and the other parts of his *body* exposed in the hot chamber which was at 30° C

Energy metabolism was then measured Results are shown in Table 5 3 from which it is observed that the rise of BM is less than 5 per cent Taking into account the restless state during that period it is clear that there is definitely no increase

This experiment shows that a moderate decrease in room temperature does not affect normal BM level as long as the body is kept in a warm comfortable condition

E. Cold Tolerance and Skinfold thickness

Various biochemical and physiological measurements have been conducted in our artificial-climate chamber for the purpose of finding a measure of tolerance to cold applicable to every individual From these experiments we have found that the *ratio of rise of energy metabolism is most nearly* proportional to the occurrence of shivering under the cold

However as measurement of energy metabolism is complicated and difficult in order to find a more *simplified measure we attempted to obtain the correlation* between the energy metabolic rate under the cold and more than twenty different biometrical morphological measurements (Suzuki 1957e) As a result we found skinfold thickness to be most worthy of consideration as a standard for the measurement of tolerance

The results are shown in Table 5 4 from which it is seen that while the rise of metabolism in subjects Nos 1–4 was markedly higher than that in subjects Nos 5–7, *the total value of skinfold thickness* measured at six locations on the body was only 35 mm in the former group while in the latter it exceeded 40 mm

Let us now consider the women divers of Japan *whose job is to dive naked into the cold sea to collect* marine plants, shell fish etc. This work begins rather early in the year—in early spring Only young, healthy women in an adequate state of nutrition are engaged in this occupation, and their thick subcutaneous fat may give practical support to our findings (Fig 5 6)

As *further supporting evidence* the increase in body weight in winter may be given Our data reveal that the chief cause of this increase is increased skinfold thickness (Suzuki 1957*f*) This is described as follows

If the average skinfold thickness of the whole body surface is assumed as approx 2 mm the increase in volume was—

$$0\ 2 \times 16\,000 = 3\,200 \text{ ml}$$

a value almost equivalent to the increase *in body* weight. This fact fully corresponds with the abovementioned small rise of energy metabolism in winter due to tolerance to cold

TABLE 5 4

Rise of Energy Metabolism and Skinfold-thickness

Subjects	Extra heat production above BM (per cent)	Skinfold thickness measurements at *six points* Sum (mm)
No 1	56 3	35 5
No 2	48 0	35 2
No 3	43 8	35 5
No 4	33 0	34 0
No 5	19 6	48 5
No 6	15 2	43 5
No 7	9 1	41 0

TABLE 5.5

Skinfold thickness of the Same Subjects (Farmers) in Summer and Winter

Location	Males (13)		Females (9)	
	Summer	Winter	Summer	Winter
Chest (mm)	3	6	8	10
Abdomen (mm)	3	6	8	14
Knee (mm)	4	4	7	6
Back (mm)	6	7	11	11
Upper arm (mm)	3	3	9	10
Sum (mm)	19	28	42	52
Body weight (kg)	52 0	54 2	47 1	40 7



Fig 56 Japanese Women Divers

III SPECIFIC DYNAMIC ACTION

Our experimental studies on specific dynamic action (SDA) for Japanese were conducted on such problems as the following—

(1) Amount of SDA for the usual Japanese meals

(2) Difference of SDA from the composition and amount of foods

(3) Availability of SDA for our daily life

(4) Some problems of theoretical interest

Results of the experiments are summarized as follows—

(1) Amount of SDA for customary Japanese food (the calorie ratios of carbohydrate protein and fat to total calories are 75 per cent 13 per cent and 12 per cent respectively) was 7 6 per cent of the calories ingested in food (Suzuki *et al* 1951*b*)

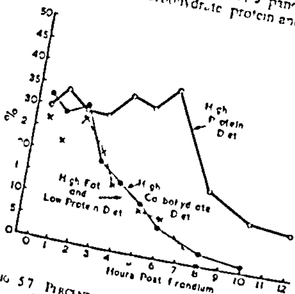


Fig 5 7 Percentage of Extra Calories Production by SDA (Subject S N)

(2) High protein diet containing 40 per cent of protein showed the largest SDA (16 17 per cent of the ingested calories) High fat diet containing 40 per cent of fat had on the contrary the least SDA viz. 6–7 per cent of the ingested calories (Fig 5 7)

In any diet however the amount of SDA is directly proportional to the ingested calories of food as seen in Fig 5 8 (Suzuki *et al* 1952*b*)

As it has been a general rule in Japan to estimate the amount of SDA as 10 per cent of the BM consequently body size would be a function of SDA However from the results of the above experiments it appears that SDA should rather be correlated with the amount of food taken in and it should therefore be considered as a function of activity as well as body size

(3) Regardless of food composition SDA cannot be utilized for the energy of muscular work from the fact that the total extra calories is the sum of the calorie increase produced by SDA and that required for muscular work as shown in Fig 5 10 (Suzuki *et al* 1953*a*)

But SDA can be utilized as a heat source for maintenance of body temperature in a cold environment and it is particularly effective in the high protein diet (Suzuki *et al* 1955) The extra calories produced by cold stimulus are very much spared by the ingestion of food as seen in Fig 5 11

(4) The distinctive nature of SDA derived from protein was that it was not only much greater in quantity than that from carbohydrate or fat but it also showed a secondary increase around the fifth hour after the meal which was always followed by a simultaneous increase of urinary nitrogen The initial increment of energy metabolism immediately after the meal is regarded as coming from digestive action since its amount is scarcely influenced by the kind of meal But the secondary increment which is never found in high-carbohydrate or high fat meals is supposed to be the actual SDA derived from protein In order to separate the two kinds of increment we

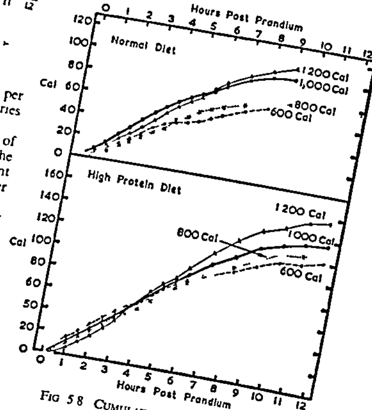


Fig 5 8 Cumulative Extra Calories

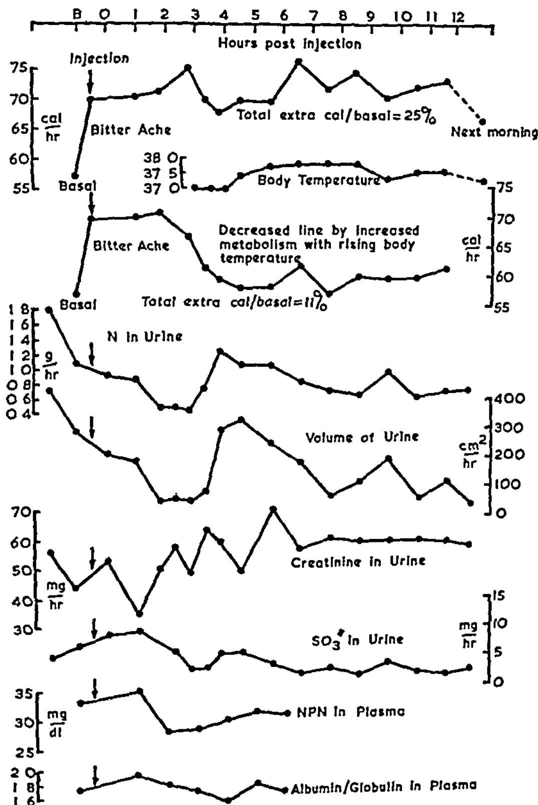


FIG 5.9 ENERGY METABOLISM AFTER SUBCUTANEOUS INJECTION OF BLOOD PLASMA (DRY PLASMA 50 G) (SUBJECT S.S.)

examined the influence of subcutaneous administration of protein which was not followed by the action of digestive organs (Suzuki *et al* 1952*a*) This experiment suggested that there was no secondary increment when the protein administered subcutaneously was not catabolized but was stored in the body These results (Fig 5 9) show that the actual SDA of protein comes from the catabolism of protein in the body

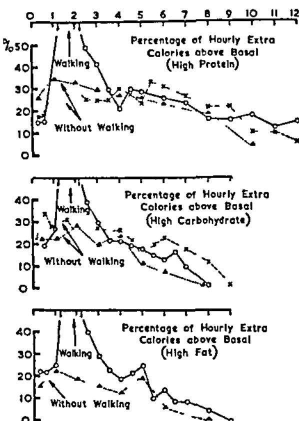


FIG 5 10 SDA AND ENERGY FOR MUSCULAR WORK

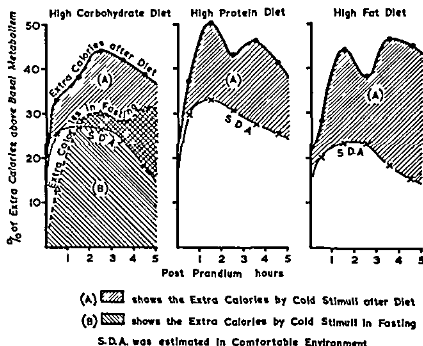


FIG 5 11 HOURLY EXTRA CALORIES ABOVE BASAL METABOLISM IN COLD ENVIRONMENT IN FASTING AND POST PRANDIUM (SUBJECT S S)

IV SLEEP

The results of many studies on the energy metabolism during sleep carried out in the past are not always in agreement Most of the foreign investigators have reported the value to be 0–20 per cent less than that of the BM while a Japanese author has reported it to be 30 per cent lower than the BM

In order to obtain a reliable value for the estimation of Japanese calorie requirements the energy metabolism during sleep throughout the night was measured on three young subjects twice in each season (Suzuki Nagamine and Kitagawa 1950 Suzuki *et al* 1951*c* Suzuki *et al* 1952*c d e*)

The average ratio of sleeping metabolism to BM is 94 per cent for each subject as seen in Fig 5 13 This figure shows the seasonal change of basal and sleeping metabolism for three subjects At a glance it is noticed that the seasonal fluctuation of sleeping metabolism runs exactly parallel with that of BM

We can conclude consequently that for the average ratio of sleeping to basal metabolism throughout a year 94 per cent is a reliable value for every season

As an unexpected result we found that the range of variation of metabolism during sleep was rather wider than we had supposed This variation was ±5 per cent unless the subjects moved or talked in their sleep when the variation amounted to ±10 per cent It seems to us that this variation has a rather wider range than that of BM This means we suppose that the reflex of the autonomic nervous system—subcortical reflex—appears easily in the resting state of the cortex

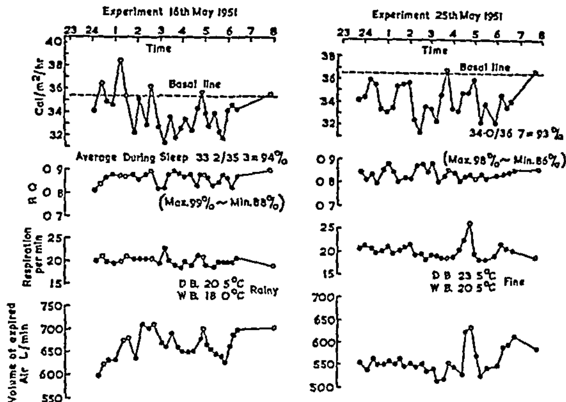


FIG 5 12 METABOLISM DURING SLEEP (SUBJECT S N)

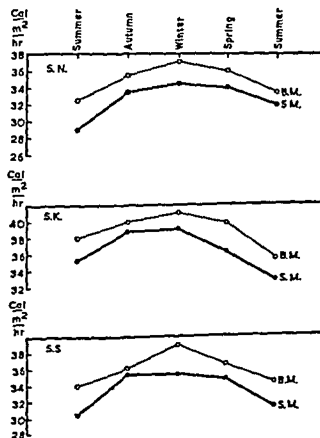


FIG 5 13 SEASONAL FLUCTUATION OF SLEEPING METABOLISM

V MENSTRUAL CYCLE

Whether there is a regular fluctuation in female basal metabolism related to the menstrual cycle is a problem still remaining undecided despite the efforts of several Japanese investigators Some of them have observed fluctuations in BM but they could not agree upon a general rule

However as regards body temperature a physiological rise occurring immediately after ovulation and continuing until the next menses has been confirmed in most women in a healthy condition In order to find a general rule for the fluctuation of BM the following experiment was carried out in the hope that it might provide an easier method of investigating BM by correlation with body temperature

The daily metabolism of two girls aged 21 and 23 was measured for a 38 day period (Suzuki *et al* 1953*b*) Body temperature was taken under the tongue with a mercury thermometer

The results were as follows—

(1) Curves of BM and body temperature run fairly parallel both showing a rise in the latter half of the menstrual cycle

(2) The correlation coefficients between BM and body temperature were in the range of 0 5–0 7 As the BM is usually influenced by a number of different factors these correlations would therefore indicate that BM runs parallel with body temperature

(3) Table 5 6 gives a comparison of average BM in the first and second half periods In subject No 1 the average BM in the second half was 4 per cent higher than in the first half and in subject No 2 2 per cent higher

TABLE 5 6
Comparison of Averages in First Half and Second Half of Menstrual Cycle

Subject	No 1		No 2	
Period	First half	Second half	First half	Second half
BM (Cal/hr/sq m)	31 1	32 2	31 2	31 8
Body temperature (F)	97 8	98 0	98 1	98 6
Pulse (per min)	62	64	58	58

(4) Maximum deviations from the arithmetic mean of the BM during 38 days were ±7 per cent and ±6 per cent in the two young women observed From this deviation it may be considered that the individual variation of female metabolism is somewhat larger than that of male BM owing to the influence of the sexual cycle

VI BODY COMPOSITION

For the purpose of clarifying the correlation between body composition and BM and also to obtain a comparison between Japanese and Westerners we have carried out body composition measurements on Japanese (Suzuki 1957*a b*)

Methods of evaluation were—

(1) Antipyrine space method body water measured by antipyrine

(2) Body density method body density measured by immersion in water and residual air

(3) Skinfold thickness method by a Minnesota type caliper

Table 5 7 shows the average values obtained from measurements made on thirty subjects male laboratory research workers

Table 5 8 gives correlation coefficients of body composition and BM

From these correlations it is seen that fat free mass measured by densitometry and body surface area is most closely related to BM followed by fat free mass measured by hydrometry, skinfold thickness and

TABLE 5 7
Body Composition

		Maximum	Minimum	Average
Age (years)		43	21	29 5
Body weight (kg)		77 9	45 7	56 49
Body surface area (sq m)		1 946	1 470	1 644
BM (Cal)	(per hr)	72 8	49 2	57 78
	(per hr/sq m)	37 4	31 6	35 1
	(per hr/kg)	1 ·05	0 863	1 0·99
Body water (per cent)		64 5	46 8	57 6
Body fat (per cent)	(1)	34 8	10 2	19 7
	(2)	22 8	4 5	13 0
Fat free mass (kg)	(1)	52 8	40 ·	44 8
	( )	60 0	4 2	48 7
Body density		1 0903	1 0574	1 0725
Sk nfold thickness—sum of 5 parts (mm)		64	11	29 1

(1) Behnke Fat (per cent) = 100−*A* 0 718
(2) Behnke Fat (per cent) = 5 548/*D*−5 044
A = Body water percentage *D* = Body density

TABLE 5 8

Correlation Coefficients between BM and Body Composition

BM (Cal/hr)	Fat free mass (densitometry)	0 874
BM (Cal/hr)	Body surface area	0 872
BM (Cal/hr)	Fat free mass (hydrometry)	0 841
BM (Cal/hr/kg)	Skinfold thickness/kg	−0 795
BM (Cal hr/kg)	Body density	0 780
BM (Cal hr)	*Body weight*	*0 6 6*
Body density	Skinfold thickness/kg	−0 681
Body fat (densitometry)	Skinfold thickness/kg	0 882
Body fat (hydrometry)	Skinfold thi kness/kg	0 862

body density while body weight is the least closely related

Of these methods, skinfold thickness was measured without the least difficulty having a high correlation of 0 88 with body fat (densitometry) and 0 86 with body fat (hydrometry) furthermore its close correlation with BM namely −0 8 deserves special attention

In addition to this Table 5 9 shows the close relationship between body density and skinfold thickness at all locations on the body from which a high correlation is observed particularly in the case of the *back chest abdomen and upper arm.*

TABLE 5.9

Correlation between Body Density and Skinfold thickness

		Sportsmen, weight lifters	Laboratory research workers
Body density	Chest SFT	−0 6[illegible]	−0 7[illegible]3
Body density	Back SFT	−0 813	−0 64
Body density	Upper arm SFT	−0 693	−0 651
Body density	Abdomen SFT	−0 79	—
Body density	Side-abdomen SFT	−0 704	−0 570
Body density	Knee SFT	−0 661	−0 531
Body density	Sum of above	−0 775	−0 691

Table 5 10 presents average skinfold thickness values of Japanese adults both male and female From this it is observed that the average value for Japanese males is approximately 50 per cent lower as compared with Westerners although the difference is not remarkable in the case of females (Suzuki 1958)

TABLE 5 10

Average Skinfold thickness of Adults

Location	Males			Females	
	Sportsmen weight lifters (30) 19–22 yrs	Laboratory research workers (14) 29 5 yrs	Aged (25) 60–69 yrs	University girls (33) 22 yrs	Aged (26) 60–69 yrs
Chest	5 9	6 5	5 0	13 0	8 0
Upper arm	6 7	4 1	4 0	20 5	9 0
Back	9 2	7 8	7 0	15 0	7 0
Abdomen	6 9	—	6 0	21 5	9 0
Side-abdomen	6 7	6 2	5 0	19 3	5 0
Knee	7 1	4 6	6 0	21 6	7 0
Average	7 1	5 9	5 5	18 5	7 5

VII. POST WAR UNDERNUTRITION

Most of the Japanese people, especially those living in the cities, had suffered from undernutrition in consequence of the food shortage during and after the Second Great War This resulted in the under growth of the younger generation as well as increased mortality and morbidity As all these symptoms seemed to come from depression of bodily vitality we have followed the recovery process of the depressed BMR of Japanese

For instance let us take the average BM values of male subjects selected from our labora ory in 1949 These values were 34 5 Cal sq m/hr in spring and

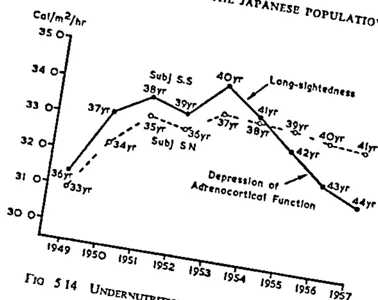


FIG 5 14 UNDERNUTRITION AND BASAL METABOLISM

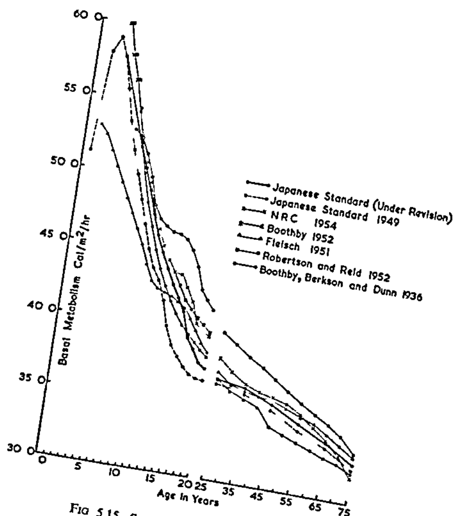


FIG 5 15 STANDARDS OF BASAL METABOLISM

32 0 Cal/sq m/hr in summer being approximately 6 per cent lower than 36 4 the average normal value of the Japanese In order to confirm the state of recovery thereafter we measured our own BM values monthly from that time (Suzuki Nagamine and Oshima 1952*f* Suzuki *et al* 1954*d*) Fig 5 14 illustrates monthly average BM values for each year

As seen in the figure BM levels seem to show signs of recovery in autumn 1949 and return to their normal levels in 1951 Since then subject S N has maintained his normal level while in another case (subject S S) there was a fall in BM values from 1954 which is believed to be due to ageing symptoms such as long sightedness and depression of the adrenocortical function appearing in parallel with the decline in BM values

Likewise, the BM of most Japanese showed a decrease of approximately 10 per cent, which was attributable to the food shortage both during and immediately after World War II

Recovery from this unsatisfactory state has been observed since the latter half of 1949 On the other hand taking into consideration the improved food situation from the latter part of 1948 we found that 1–2 years are required for recovery from such under nutrition lasting for a few years

Moreover despite the fact that two of these subjects were self fed on a high protein diet comprised of protein (120 gm per day) for a 1 month period in autumn 1949 only a slight rise in their BM levels could be observed during this high protein diet period.

It is thus quite conceivable that long term under nutrition has an extremely severe influence on the living organism

VIII. STANDARD VALUES OF BASAL METABOLISM AND BODY SIZE FOR JAPANESE

The standard BM values (per sq m per hr) for Japanese previously in use (adopted in 1949) are now under revision in the hands of the Committee on Calorie Requirements on the ground that numerous data have been presented since that time

Although the standard values for the Japanese in the past were considerably lower in comparison with Westerners particularly the values of those in the development stage the Committee on Calorie Requirements after investigation of this point concludes that this difference is due to the erroneous statistical methods applied in the past The Committee is accordingly contemplating a revision of standard basal metabolism values for Japanese with a view to amending the above incorrect values concurrently recording recently observed values Although these

TABLE 5 11

The Present Body Size of Japanese (1956)
(Ministry of Welfare and Health)

Age	Height (cm)		Weight (kg)	
	Male	Female	Male	Female
0	65 6	63 6	7 40	6 91
1	77 3	75 9	9 91	9 58
2	85 8	83 9	12 23	11 53
3	92 6	91 1	13 91	13 40
4	98 8	97 5	15 36	14 90
5	104 7	103 7	16 88	16 46
6	110 0	109 0	18 58	17 83
7	115 5	114 6	20 62	19 92
8	120 6	119 7	22 74	22 06
9	124 8	124 5	24 58	24 12
10	129 0	129 6	26 94	27 18
11	134 4	135 9	29 76	30 70
12	139 5	140 7	33 06	35 12
13	144 6	144 9	36 80	38 94
14	152 1	149 1	43 20	43 32
15	157 8	150 9	48 64	45 68
16	160 2	151 5	51 84	47 86
17	161 7	151 2	53 60	49 50
18	162 6	151 8	54 22	49 78
19	162 0	151 8	54 94	49 84
20	162 3	151 8	55 44	49 38
21	162 9	151 8	55 72	49 80
22	162 6	150 9	55 86	49 16
23	162 9	151 2	56 86	49 60
24	162 0	150 6	54 94	48 72
25	162 3	150 9	54 90	48 96
26–29	161 7	150 9	55 00	48 82
30–39	161 4	149 7	55 50	48 70
40–49	159 9	148 5	55 32	49 02
50–59	158 7	146 7	54 08	47 64
60–69	156 6	143 7	51 52	44 38
70–	154 2	140 7	48 78	41 60

revised values are yet not rigidly determined a trend curve is presented in Fig 5 15

The body size of Japanese has increased since 1900 year by year For instance an increase of approximately 1 cm has been observed in the stature of Japanese in each decade

This rising trend has however been interrupted by the food shortage situation that arose from World War II resulting in a conspicuous decline in stature which was most apparent in adolescence i e 4–5 cm

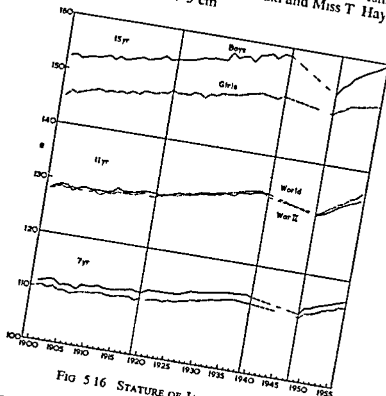


FIG 5 16 STATURE OF JAPANESE PUPILS

A few years after the war in accordance with the favourable turn of the food situation the stature status of the Japanese also concomitantly began to reach its former level Moreover as this tendency increased with the passage of time the present bodily stature of the Japanese surpasses its prewar level (Fig 5 16)

In addition to this the present body size of the Japanese is illustrated in Table 5 11

Great indebtedness and gratitude are here acknowledged to Dr S Nagamine Miss S Oshima Miss K Yamakawa Dr S Kawada Mr T Kuga Mr S Kitagawa Miss Y Ishibashi Dr T Tezuka Mr H Suzuki and Miss T Hayakawa for their able collaboration and strenuous efforts towards the accomplishment of this work Thanks are also due to Mr H Suzuki for the translation into English and to Miss S Oshima for the preparation of the figures and tables in this paper

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6

Nutritional Significance of Vitamin B_{12}

I Metabolic Functions in Experimental Animals

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II Mechanism of Absorption of Vitamin B_{12}

III Vitamin B_{12} in Health and Disease

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Contents

Following the crystallization of vitamin B_{12} a decade ago numerous investigators have studied the physiological effects of this vitamin in attempts to elucidate its mode of action and to assess its usefulness in treatment of disease In this communication some of the highlights of these investigations will be presented The major areas which will be considered are—

(1) the metabolic function of vitamin B_{12} as it appears from *in vivo* and *in vitro* studies with animals

(2) the mechanism of absorption

(3) the role of vitamin B_{12} in health and disease

Only the first of these topics will be considered in Part I The second and third portions of this review will deal with the mechanism of vitamin B_{12} absorption and the role of this vitamin in health and disease

I METABOLIC FUNCTIONS IN EXPERIMENTAL ANIMALS

A deficiency of vitamin B_{12} becomes manifest in a variety of structural and functional breakdowns depending upon the species under study The classical changes seen in the pernicious anaemia of man have not been reproduced *in toto* in any experimental animal Similarly studies on the biochemical changes associated with vitamin B_{12} deficiency in animals have led down a multidivergent path To date no specific metabolic function can be ascribed to vitamin B_{12} Indeed it is difficult to imagine that a single mechanism could account for the great variety of metabolic effects attributed to the vitamin however many of those may very well prove to be of a secondary nature

A Protein Metabolism

1 Incorporation of Amino Acids into Protein

Within recent months several reports of an effect of vitamin B_{12} upon protein synthesis have come from Wagle Mehta and Johnson (Wagle and Johnson 1957 Wagle Mehta and Johnson 1957 1957*a* 1958) at the University of Illinois Since this work may presage the definition of vitamin B_{12} function these papers will be reviewed first The older studies relating vitamin B_{12} with protein and amino acid metabolism will then be considered in the light of these more recent findings

Wagle and co-workers studied the effect of vitamin B_{12} upon the incorporation of radioactively labelled amino acids into proteins by *in vivo* and *in vitro* techniques Vitamin B_{12}-deficient and supplemented baby pigs and rats were injected with [3 ^{14}C]serine After 4 hours the vitamin B_{12} supplemented animals had incorporated about 50–60 per cent more radioactive material into liver protein than had the deficient animals This difference was somewhat less than the difference in weight between the deficient and supplemented animals (Wagle Mehta and Johnson 1958 Neumann Johnson and Thiersch 1950) and may be simply an indication of the growth rates at the time of the experiment

Wagle *et al* (1958) also employed the *in vitro* method devised by Keller and Zamecnik (1956) They used an enzyme system which contained the microsomes and supernatant fractions of a tissue homogenate fructose diphosphate adenosine triphosphate guanosine triphosphate and a labelled amino acid Protein synthesis was measured by the amount of radioactivity present in the proteins at the end of the incubation period

For their *in vitro* experiments Wagle *et al* used enzyme preparations from liver and spleen of vitamin B_{12}-deficient and supplemented rats Incorporation of [Me ^{14}C]methionine into protein was much greater in preparations from vitamin B_{12} supplemented rats than in those from deficient rats When vitamin B_{12} was added in millimicrogramme quantities (within the physiological range of liver and spleen vitamin B_{12} concentrations) to the *in vitro* system from deficient animals the incorporation of either [Me ^{14}C]methionine or [2 ^{14}C]alanine was increased two- to threefold nearly to the rate of incorporation observed with animals fed vitamin B_{12} In preparations from the control animals only slight stimulation of amino acid incorporation was observed with the *in vitro* addition of vitamin B_{12}

The amount of [3 ^{14}C]phenylalanine incorporated into protein in the presence of a pool of eighteen amino acids was appreciably higher than the incorporation of either alanine or methionine However the amount of [3 ^{14}C]phenylalanine incorporated in the absence of the amino acid pool was not determined and this largely precludes drawing conclusions about the effect of an amino acid pool Keller and Zamecnik found no increase in the incorporation of ^{14}C labelled amino acids into protein in the presence of an amino acid pool (1956)

Intracellular distribution of vitamin B_{12} was determined by Wagle Mehta and Johnson (1958) in a single normal rat injected with 1 μc of ^{60}Co-labelled vitamin B_{12} After 6 hours the distribution in percent of radioactive vitamin B_{12} in the liver was as follows—nuclei and unbroken cells 11 4 mitochondria 13 6 microsomes 40 5 supernatant fluid 22 5 It is of

interest that the sources of enzymes active in incorporating amino acids into proteins (the microsomes and supernatant) contained the greatest amounts of radioactive vitamin B_{12}

These studies raise far more questions than they answer, regarding both theoretical implications and the control and expansion of these investigations The *mechanisms involved in protein* synthesis are largely unknown From these studies there is no indication as to whether there was net synthesis of protein during the incubation period or whether the actual amount of radioactivity present at the end of the incubation period represented amino acid exchange or formation of new protein molecules The amount of protein and free amino acids present in one system initially may be of importance to the results apart from the amount of enzymes and cofactors specifically involved in protein synthesis It is reasonable to expect that these factors would be quite different between vitamin B_{12}-deficient and -supplemented animals, possibly owing to conditions associated with the deficiency and not directly related to vitamin B_{12} *per se* Data from some type of inanition control animals receiving vitamin B_{12} would add greatly to the value of these data

The diet which the rats received did not support optimal growth even when supplemented with vitamin B_{12} (Wagle Mehta and Johnson, 1958) At 10 weeks of age the rats (Sprague-Dawley strain) averaged only 203 g in weight about 100 g less than one would expect This poor growth was probably due to the high amount of lactose in the diet (22 per cent) The use of lactose in exaggerating a vitamin B_{12} deficiency was recommended by Cuthbertson and Thornton (1952) however their experiments lasted for only 4 weeks For studies of vitamin B_{12} function such as the above it would be desirable to use rats born from mothers fed a vitamin B_{12}-deficient diet rather than to resort to some type of stressful diet.

The most significant result found in the work of Wagle and co-workers is the marked effect of vitamin B_{12} added *in vitro* to the system from the deficient animal This is doubly significant since the same effect was observed in preparations from both liver and spleen In almost no other studies where a low level of some enzyme has been found in a vitamin B_{12}-deficient organism has a stimulatory effect been obtained upon addition of the vitamin *in vitro* (see below) If vitamin B_{12} plays some major role in the synthesis of protein, the implications in almost all phases of metabolism are readily apparent

2. Protein and Amino Acid Utilization

During the past decade numerous investigators have attempted to relate vitamin B_{12} function to protein and amino acid metabolism through many parameters of response In some of the early studies it was found that *increasing the protein content of the* diet increased the severity of the vitamin B_{12} deficiency Hartman Dryden and Cary (1949) showed that increasing the casein from 25 to 45 or 65 per cent of the diet caused *progressively poorer growth of rats depleted in factor* X (vitamin B_{12}) Crystalline vitamin B_{12} overcame the poor growth under these conditions however larger quantities of vitamin B_{12} were required to maintain a given rate of growth as the *casein content of the* diet was increased Similar growth *responses have* been reported with mice by Bosshardt, Paul and Barnes (1950) and with young chicks by Hill and Branion (1952) and Spivey Briggs and Ortiz (1954) In studies with hens Yacowitz *et al* (1952) observed a more rapid decline in hatchability of eggs laid by hens receiving a diet containing 25 per cent protein as compared with 16 per cent protein. This decline in hatchability correlated well with decreasing vitamin B_{12} content of the eggs

In a similar manner high levels of individual amino acids have caused more severe toxic manifestations in vitamin B_{12}-deficient chicks than in those that received vitamin B_{12} Menge and Combs (1950) found that addition of either 1 or 4 per cent glycine to the diet depressed the growth of vitamin B_{12}-deficient chicks and with 4 per cent glycine mortality was high These adverse effects were largely overcome when vitamin B_{12} was present in the diet. Similar effects of vitamin B_{12} were observed by Machlin *et al* (1952) in chicks fed 3 6 and 9 per cent glycine in the diet

Hsu and Combs (1952*a*) fed chicks nine different amino acids at dietary levels of 4 per cent. L Leucine and glycine caused growth inhibition in the absence of vitamin B_{12} which was overcome by incorporation of the vitamin into the diet DL Aspartic acid and L tyrosine caused growth inhibition which was partially overcome by the vitamin, whereas vitamin B_{12} was without effect on the toxicity produced by DL alanine DL methionine or L-cystine The growth inhibitory effect of zein in the absence of vitamin B_{12} was related to the leucine content.

Attempts to influence nitrogen retention by vitamin B_{12} supplementation have been largely unsuccessful in the rat (Chow and Barrows, 1950) and in the chick (Brown, 1957) On the other hand, an effect of vitamin B_{12} upon attaining nitrogen retention has been observed in rats receiving thyroid active material (Rupp Paschkis and Cantarow 1951 Cheng and Thomas, 1952) More recently Henry and Kon (1956) could find no increase in the biological value of protein with vitamin B_{12} supplementation.

The carcass composition of experimental animals has not yielded data that would indicate a function of vitamin B_{12} in protein utilization. Ling and Chow

(1952) fed vitamin B_{12}-depleted weanling rats a soybean protein diet *ad libitum* Control animals *received the same diet ad libitum* plus vitamin B_{12} by injection After a 5 week period significant differences in growth were obtained between the deficient and supplemented animals The percentage protein in the carcass and the muscle glycogen were unaffected by vitamin B_{12} deficiency In the deficient animals the fat content of the carcass was reduced from 16 6 per cent in the supplemented controls to 4 3 per cent and the water content increased from 58 6 to 67 0 per cent Similar but less pronounced effects on fat storage have been reported by others in the rat (Black and Bratzler 1952 Knoebel and Black 1952) and in the mouse (Mirone 1953)

3 Concentrations of Nitrogenous Components

The vitamin B_{12} status of the animal has been found to have a profound effect upon the level of various nitrogenous constituents of the blood under certain conditions Schultze (1949) and Bruemmer O Dell and Hogan (1955) observed high levels of urea in the blood of newborn rats whose mothers had received a soy bean protein diet free of vitamin B_{12} Schultze found that this uraemia which was frequently fatal could be abolished by injection of vitamin B_{12} into the young rat In the growing chick a deficiency of vitamin B_{12} has been found to increase the blood levels of non protein nitrogen amino nitrogen urea and creatinine (Hsu and Combs 1952 1952*a*) The level of uric acid in chick blood was not affected by vitamin B_{12} deficiency however vitamin B_{12} supplementation of chicks receiving a toxic level of glycine (Machlin *et al* 1952 Hsu and Combs 1952*a*) or of L leucine or zein (Hsu and Combs 1952*a*) increased the blood uric acid level The latter finding was interpreted as indicating a function of vitamin B_{12} in the formation of uric acid in the chick

The influence of vitamin B_{12} on the level of free amino acids in the blood is not well defined Charkey *et al* (1950) reported slightly higher levels of each of seven amino acids in the blood of chicks fed a vitamin B_{12}-free diet as compared with supplemented controls In a second study (Charkey Kano and Anderson 1954) wherein the effect of fasting was also studied the response to vitamin B_{12} was variable No indication of variability within groups was presented and the growth of chicks supplemented with vitamin B_{12} in each experiment was close to that of chicks receiving the deficient diet In addition Richardson Blaylock and Lyman (1953) found significantly lower levels of free arginine lysine methionine tryptophan, and valine in the plasma of chicks deficient in vitamin B_{12} as compared to those receiving the vitamin. In this study the growth of the two groups of chicks was significantly different Salander and Patton (1952) studied the level of free amino acids in the liver of chicks deficient in and supplemented with vitamin B_{12} They found measurable amounts of alanine, glutamic acid and taurine, which were present in similar amounts in livers from both groups of chicks They also found a peptide composed of aspartic acid glutamic acid glycine cysteine and phosphorus which was present in considerably higher quantities in the vitamin B_{12} supplemented chicks

The total plasma proteins and the total albumin have been found to be lowered in vitamin B_{12}-deficient chicks (Hsu Stern and McGinnis, 1953) Total globulin was lowered in deficient females but not in males In a double deficiency of folic acid and vitamin B_{12} in rats the total serum proteins serum albumin α globulin and γ-globulin were reduced (Mulgaonkar and Sreenivasan 1957) The specific effect of vitamin B_{12} cannot be determined from this study however

4 Enzyme Concentrations

A summary of the reported effects of vitamin B_{12} deficiency upon the level of various enzymes in tissues of experimental animals is presented in Table 6 1 In most instances where a change occurred it was toward a lower level in the deficient state There were two exceptions however In the first choline oxidase of rat liver the authors suggested that at least part of this increase may have been due to an artifact of their experimental conditions In the case of arginase activity of the liver in newborn rats from vitamin B_{12}-deficient mothers the effect was marked (Liener and Schultze 1950) The enzyme level was normal immediately following birth but rose rapidly as soon as the young nursed The increase in arginase correlated well with an increase in uraemia both could be prevented by injection of vitamin B_{12} into the young when they were born Many of the observations in Table 6 1 are single reports and it is hoped that the studies will be repeated in other laboratories The enzyme change that has been studied most extensively is that of transmethylase of rat liver Several groups of workers have observed a decrease in transmethylase activity in livers of vitamin B_{12}-deficient rats however Stekol *et al* (1957) have failed to find an effect of vitamin B_{12} upon this enzyme It is of interest also, that a decrease in transmethylase activity was not observed in the livers of deficient pigs and chicks

In many of the experiments vitamin B_{12} was added *in vitro* and had no effect on enzyme activity except for transmethylase (Ericson *et al* 1956) and the protein synthesizing systems (Wagle and Johnson 1957, Wagle Mehta and Johnson 1957 1957*a* 1958) The negative *in vitro* effects have been considered evidence for an effect of vitamin B_{12} upon synthesis of

some portion of the enzyme Indeed the positive effect of vitamin B_{12} in the transmethylase system was considered to be indirect since vitamin B_{12} could not be detected in highly purified cofactor preparations for the enzyme (Ericson *et al* 1956) and also there emerges Part of the difficulty probably arises from the variety of functions in which vitamin B_{12} seems to have a role This diversity may result in preferential shunting of vitamin B_{12} for specific functions in varying degrees of efficiency which may account for some of

TABLE 6 1

Effect of Vitamin B_{12} Deficiency upon Levels of Various Enzymes in Animals[1]

Enzyme system	Tissue analysed	Effect of B_{12} deficiency[2]	Reference
Amino acid incorporating	Rat liver	−	Wagle and Johnson (1957) Wagle Mehta and Johnson (1957 1957*a* 1958)
	Rat spleen	−	*ibid*
D-Amino acid oxidase	Chick liver	0	Williams Nichol and Elvehjem (1949)
Arginase	Rat liver newborn	+	Liener and Schultze (1950)
Carboxylase	Rat liver	−	Iwamoto Hayashi and Sahashi (1957)
	Rat thigh muscle	−	*ibid*
Catalase	Chick liver	0	Williams Nichol and Elvehjem (1949)
Choline oxidase	Rat liver	+	Williams *et al* (1953*a*)
	Rat liver newborn	0	O Dell *et al* (1955)
	Chick liver	0	Gillis and Young (1951)
Cytochrome oxidase	Rat liver	−	O Dell *et al* (1955)
	Rat liver newborn	−	*ibid*
	Rat brain newborn	*0*	*ibid*
	Rat kidney newborn	0	*ibid*
	Rat skeletal muscle newborn	0	*ibid*
Dehydrogenases (lactic glutamic succinic butyric choline)	Rat liver	−	Murthy Desikachar and Swaminathan (1956)
Deoxyriboaldolase	Rat liver	−	Wong and Schweigert (1957)
Endogenous respiration	*Rat liver*	−	*Williams et al* (1953*a*)
	Rat liver newborn	0	O Dell *et al* (1955)
	Chick liver	0	Williams Nichol and Elvehjem (1949)
Succinoxidase	Rat liver newborn	−	O Dell *et al* (1955)
Transmethylase	Rat liver	−	Oginsky (1950) Williams *et al* (1953 1953*a*) Ericson *et al* (1956) Mistry *et al* (1955)
	Rat liver	0	Stekol *et al* (1957)
	Pig liver	0	Mistry *et al* (1955)
	Chick liver	0	*ibid*
Xanthine oxidase	Rat liver	−	Williams *et al* (1953 1953*a*)
	Rat liver	*0*	Westerfeld and Richert (1952)
	Chick liver	*0*	*Williams Nichol and Elvehjem* (1949)

[1] All tissues were from growing animals unless otherwise stated
[2] + indicates increase of deficient over supplemented animals − decrease and 0 no change

appeared to be no direct relationship between vitamin B_{12} content of the enzyme and enzymatic activity (Ericson, Williams and Elvehjem, 1955)

In spite of the diversity of approach and the tremendous amount of work which has been expended in an attempt to relate vitamin B_{12} function to protein and amino acid metabolism, no clear-cut picture the negative results that have been observed. The various stresses, such as thyroid feeding, that have been widely used to accentuate vitamin B_{12} deficiency may have exerted effects apart from that on the *deficiency state alone.*

In experiments where changes in concentration of nitrogen-containing compounds have been altered by

vitamin B_{12} there remains the difficult problem of determining whether the vitamin exerts its effect on input or output of the material being studied Although *in vitro* enzyme studies may provide valuable clues as to the mode of action of vitamin B_{12} these changes must be related to function in the intact cell and animal

5 Binding of Vitamin B_{12} by Protein

If vitamin B_{12} acts as a cofactor for some enzyme the manner in which the vitamin is attached to the apoenzyme is of interest It became apparent in the early work with intrinsic factor that vitamin B_{12} could readily be bound by a variety of proteins that had no

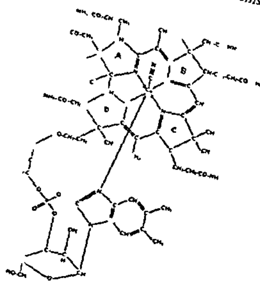


Fig 6 1 Structure of Vitamin B_{12}

intrinsic factor activity The present status of our knowledge regarding vitamin B_{12}–protein attachments and configurational arrangements has recently been reviewed by E Lester Smith (1958) Evidence to date supports the view of a break in the nucleotide–cobalt bond of vitamin B_{12} and of protein attachment (for example through the nitrogen of a histidine residue) to the cobalt atom (see Fig 6 1) Smith (1958) visualizes the nucleotide on the end of its flexible chain being free to wrap itself around part of the protein molecule so that for example oppositely charged and suitably situated sites on the two molecules are in juxtaposition This part of the molecule (the nucleotide) probably represents as it were just the end of a line of hooks by which the vitamin attaches itself to its appropriate biological environment

In recent studies intrinsic factor has been shown to increase the uptake of vitamin B_{12} by liver slices *in vitro* (Miller and Hunter 1957 Latner and Raine 1957 Herbert 1958) The physiological significance remains to be shown however it is possible that a substance similar to or identical with intrinsic factor may mediate the transport and intracellular disposition and activity of vitamin B_{12} This will undoubtedly be an interesting area of future research

From the above discussion no inference is intended that the function of vitamin B_{12} is definitely established as being within the area of protein metabolism Unfortunately a detailed consideration of all aspects of the very big field of vitamin B_{12} activity is beyond the space limitations of the present review Only the high points of the remaining topics will be given and reliance will be placed on other reviews in these areas

B Nucleic Acid Metabolism

In vitamin B_{12} deficient animals a number of workers have observed decreased levels of ribonucleic and desoxyribonucleic acids (see reviews by Wong and Schweigert 1956 O Dell and Bruemmer 1957) as well as deranged ratios of the two (Bruemmer O Dell and Hogan 1955) Recent studies have indicated that vitamin B_{12} affected the incorporation of phosphorus into nucleic acid under some conditions (O Dell and Bruemmer 1957 Venkataraman and Barnum 1957) Wagle *et al* (1958) have reported studies in which the incorporation into liver nucleic acid of various radioactive precursors was determined in chicks and pigs deficient in and supplemented with vitamin B_{12} The vitamin had no effect upon incorporation of [^{14}C]formate [^{14}C]formaldehyde [2 ^{14}C]glycine [3 ^{14}C]serine [Me ^{14}C]methionine or [^{14}C]glucose into either RNA or DNA Similarly vitamin B_{12} had no effect on incorporation of labelled glycine methionine formate or serine into the allantoin excreted by the deficient and supplemented groups

C Sulfhydryl Groups

A role of vitamin B_{12} in maintaining sulfhydryl groups in a reduced state was proposed by Dubnoff (1950) Such a function would have great significance in terms of individual metabolites as well as enzymes that require sulfhydryl activation This hypothesis gained support in the finding of lowered levels of soluble sulfhydryl groups (principally in glutathione) in the blood cells (Ling and Chow 1953 Register 1954) and liver (Register 1954) of vitamin B_{12}-deficient rats Vitamin B_{12} deficiency caused an increase in the concentration of free pantothenic acid in chick liver (Yacowitz, Norris and Heuser 1951) and also an increase of coenzyme A in the liver of chicks (Boxer

Ott and Shonk 1953) and rats (Boxer *et al*, 1955) In the latter study the ratio of reduced to oxidized coenzyme A was unaffected by vitamin B_{12} status Data from recent studies have shown that vitamin B_{12} is not involved in maintaining homocysteine in the reduced state (Racker 1955 Stekol *et al* 1957) and it seems unlikely that vitamin B_{12} is directly involved in this area of metabolism

D Carbohydrate Metabolism

The relationship between vitamin B_{12} and carbohydrate metabolism has recently been reviewed by Ling and Chow (1957) Better growth responses to vitamin B_{12} have been obtained in mice (Bosshardt Paul and Barnes 1950) and rats (McCollum and Chow 1950) on diets high in carbohydrate and low in fat The blood sugar levels were found to be high in vitamin B_{12} deficient rats (Ling and Chow 1957) and chicks (Hsu and Combs 1952 1952*a*) The level of blood glucose in newborn rats deficient in vitamin B_{12} was not significantly different from non-deficient animals however the glycogen content of the liver was only 0 39 per cent in deficient animals as compared with 2 02 per cent in the controls (Bruemmer O Dell and Hogan, 1955) Deficient rats were unable to clear glucose from the blood at a normal rate during a glucose tolerance test (Ling and Chow 1954 1957) and it was possible to maintain marked hyperglycaemia in deficient rats by parenteral administration of a 25 per cent solution of glucose at 2-3-day intervals The fasting blood sugar level rose gradually during the course of the experiment from 120 mg per cent to 367 mg per cent in 24 days Injection of vitamin B_{12} largely prevented this The rise in fasting blood sugar level seen in deficient animals was accompanied by a decrease in glutathione concentration in the blood cells *In vitro* studies with red blood cells from vitamin B_{12}-deficient and supplemented rats showed decreased rates of oxygen consumption and ribose formation in cells from the deficient animals (Ling and Chow 1957) These changes were raised to normal levels by *in vitro* addition of glutathione

Further possible interrelationships between the coenzymes in carbohydrate metabolism and B_{12} deficiency were demonstrated by a marked increase in the ratio of the oxidized to reduced DPN in the livers of deficient rats as compared to that of treated rats (Chang *et al* 1958) These investigators also found that vitamin B_{12} deficiency resulted in increases of lactic and pyruvic acids This phenomenon was not observed in starved animals (Chang *et al* in press)

E. Metabolism of One-carbon Intermediates

A vast amount of experimental work has been devoted to the relationship between vitamin B_{12} and the one-carbon intermediates of biological importance This information has been reviewed (Arnstein, 1955 1957 Mistry and Johnson 1957) It is fairly certain that vitamin B_{12} is not involved in the transfer of labile methyl groups. Rather, the vitamin appears to be of importance in the reductive *de novo* biosynthesis of methyl groups from more highly oxidized precursors

F Fat Metabolism

Relatively little experimental evidence relates vitamin B_{12} directly to fat metabolism Ling and Chow found reduced quantities of phospholipids (1954) and total fat (1952) in carcasses of vitamin B_{12}-deficient rats A marked increase in the severity of the vitamin B_{12} deficiency (Spivey Briggs and Ortiz, 1954) and an increase in requirement of vitamin B_{12} (Fox Ortiz and Briggs 1956) was observed in chicks when the fat content of a corn-soybean oil meal diet was increased from 3 to 24 per cent The concentration of vitamin B_{12} in the chicks livers was unaffected by fat level of the diet In the absence of dietary vitamin B_{12} the concentration of the vitamin in the liver was essentially constant even though at 4 weeks of age the chicks receiving 3 per cent fat weighed about 70 per cent more than the chicks receiving 24 per cent fat These effects of the high fat diet upon vitamin B_{12} requirement could be abolished by supplementary methionine but not by choline (Fox Briggs and Ortiz, 1957) The data indicate that methionine and vitamin B_{12} are related in the utilization of dietary fat possibly in some way apart from the labile methyl group of methionine It appears that the lipotropic effects demonstrated with vitamin B_{12} were sometimes mediated via labile methyl group metabolism perhaps involving choline and methionine (Shils and Stewart, 1954 see reviews by Best *et al* 1953 and Forbes and Patterson 1953)

G Activity of Vitamin B_{12} as a Co-factor

The extremely small quantities of vitamin B_{12} required to satisfy the animal s need certainly point to a co-factor function The exact chemical mechanism(s) by which vitamin B_{12} could act has been recently reviewed (Smith 1958) It appears that the active site(s) may involve one or all of the three labile propionamide groups which are bonded to the porphyrin portion of the molecule (see Fig 6 1) Sterically the propionamide groups are arranged on the side of the porphyrin plane opposite the cyano group and the apoenzyme Substitution of the amide groups has resulted in compounds with anti vitamin B_{12} activity Another proposed mechanism involves reduction of the trivalent cobalt atom to the divalent state wherein it could act as a reducing agent. The

redox potential is however somewhat outside the physiological range

H Concluding Comments

Although the precise mechanism(s) by which vitamin B_{12} performs its function(s) is largely unknown the widespread ramifications of the vitamin s importance are well recognized The evidence that vitamin B_{12} may act as a co-factor in the synthesis of proteins has been reviewed Data in this area have come from one laboratory only and remain to be confirmed by others If vitamin B_{12} does play a major role in protein synthesis then the other metabolic derangements observed in vitamin B_{12}-deficient animals could be mediated via changes in apoenzyme formation This mode of action seems at this time to embody the only hypothesis of function that might account for the many metabolic effects associated with vitamin B_{12}

It is entirely possible on the other hand that vitamin B_{12} has more than one primary function in the animal organism Such a large complex molecule with a variety of functional groups could logically participate in many different types of biochemical reactions It is established that at least under some conditions vitamin B_{12} deficiency can lead to decreased formation of nucleic acid and labile methyl groups inability to utilize high levels of fat and protein reduction in levels of non protein sulfhydryl groups and derangements in carbohydrate metabolism In many of these areas the data seem to be conflicting Probably all of the factors involved have not been properly appreciated and controlled Certainly the next few years should broaden our understanding of vitamin B_{12}

II MECHANISM OF ABSORPTION OF VITAMIN B_{12}

Unlike other water soluble vitamins vitamin B_{12} given by mouth is poorly absorbed (Chow *et al* 1950 1951) In spite of numerous studies dealing with absorption of vitamin B_{12} the mechanism of absorption is not well understood and the available means of enhancing absorption are limited We shall now consider the site of absorption and also chemical and physiological factors affecting absorption

A Site of Absorption

The absorption of vitamin B_{12} by pernicious anaemia patients is practically nil unless the vitamin is co administered with intrinsic factor obtained from the gastric juice of healthy individuals The stomach is considered to be an important site for the absorption since patients with pernicious anaemia cannot absorb orally administered vitamin B_{12} because of atrophy of this organ More recently it has been shown that total gastrectomy in man (Chow *et al* 1958) and rats likewise (Swenseid *et al* 1954 Chow Quattlebaum and Rosenblum 1955 Nieweg *et al* 1956 Glass Pack and Mersheimer 1955) results in a diminution of absorption of orally administered vitamin B_{12} The absorption of these subjects can be improved upon the co-administration of intrinsic factor It is obvious that the stomach is not only an essential site for absorption of vitamin B_{12} but is a source of an agent which aids absorption However other data demonstrate that there may be more than one site of absorption of vitamin B_{12} in the gastrointestinal tract The extent to which each site may play a role is dependent on the dosage given Thus when a small dose (1–2 mcg) of radioactive vitamin B_{12} was administered by mouth to totally gastrectomized subjects a limited extent of absorption could be detected either by the urinary excretion or faecal excretion tests On the other hand when a large dose (*e g* 1 000 mcg vitamin B_{12}) was given *per os* to the same subjects the serum level was elevated to an extent greater than that of control subjects with intact stomachs given the same test dose These gastrectomized subjects responding to the 1 000-mcg dose likewise excreted considerable amounts of the orally administered radioactive vitamin B_{12} in the urine following an intramuscular injection of a flushing dose of 1 mg of unlabelled B_{12} two hours later These studies clearly indicate that the absorption of vitamin B_{12} can take place in the absence of the stomach if the dosage is sufficiently large In a like manner Conley and Krevans (1955) have been able to maintain pernicious anaemia patients in remission for several years when given oral doses in amounts of 1–3 mg

B The Fate of Orally Administered Vitamin B_{12}

The administration of vitamin B_{12} by mouth in physiologic amounts to healthy individuals results in essentially no biological activity in the urine Almost the entire dosage appears in the faeces If one assumes that the absorbed vitamin B_{12} will appear in the urine and the unabsorbed portion will be excreted in the faeces the presently available data demonstrate poor absorption of this vitamin However Okuda Grasbeck and Chow (1958) found that when a minute physiologic amount of radioactive vitamin B_{12} was given even by injection only a small portion of the radioactivity appeared in the urine and more important a big portion appeared in the faeces particularly during the sixth and fourteenth days following injection It was postulated that the injected or absorbed vitamin B_{12} travels through the liver and bile and finally is excreted in the faeces Similar findings were made by Grasbeck Nyberg and Reizenstein

(1958) in man Consequently the amount of vitamin B_{12} absorbed in healthy subjects may be much greater than that believed by many investigators

C. Factors Affecting the Absorption of Vitamin B_{12}

Three factors newly recognized which affect the absorption of vitamin B_{12} will be discussed

1 Effect of Administration in Divided Dosages on Urinary Excretion

The ^{60}cobalt labelled vitamin B_{12} in various amounts (2 0 μg 8 0 μg and 50 μg) was given by mouth to two groups of clinically healthy subjects One group received one of the above mentioned quantities in one single dose with water for rinsing The second group received the same amounts of radioactive vitamin B_{12} in four divided doses at intervals of 15 minutes apart The urinary excretion of radioactive vitamin B_{12} was taken as a measure of absorption It was found (Chow *et al*, 1958) that the administration of vitamin B_{12} in divided doses resulted in a definite increase in the radioactivity in the 24-hour urine over the group receiving the same amount of radioactive vitamin B_{12} in single doses The differences are statistically significant It was postulated that the effect of the divided doses of 8 μg or more is due to the insufficiency of intrinsic factor needed for the absorption of this large amount of vitamin B_{12} Thus when a non inhibitory intrinsic factor concentrate was used (Chow *et al* 1956) actual enhancement in the urinary excretion was observed

2 Physical State of Vitamin B_{12} Administered

Inasmuch as the relative importance of the various portions of the gastrointestinal tract for the absorption of vitamin B_{12} is poorly understood it may be expected that the physical state in which the vitamin B_{12} is to be administered may play a critical role in absorption As an illustration the absorption of vitamin B_{12} given in a gelatin capsule (Chow *et al* 1958) was compared with that given in an aqueous solution by itself or together with an agent capable of enhancing absorption Three groups of clinically healthy individuals of twelve subjects each were given *daily the following treatments*—

- Group A—100 μg of vitamin B_{12} in a hard gelatin capsule
- Group B—100 μg of vitamin B_{12} in an aqueous solution
- Group C—25 μg of vitamin B_{12} in a lipotropic elixir [1]

Serum specimens were obtained from all the subjects at regular intervals for the determination of the vitamin B_{12} activity The initial serum vitamin B_{12} levels in all three groups were statistically indistinguishable One month after treatment the serum vitamin B_{12} level was elevated significantly only in those receiving elixir (Chow Horonick and Okuda, 1956*a*) After 4 months, there was only a slight increase in Group A but marked increases in Groups B and C The elevation was more pronounced in Group C than in Group B Six months afterwards, the level of Group A was essentially the same as that of Group C after only 1 month of administration at one-quarter of the daily dose The treatment with 100 μg of vitamin B_{12} in solution for 6 months resulted in an elevation equal to the treatment of 25 μg of vitamin B_{12} in elixir for 4 months These data show the effectiveness in administering vitamin B_{12} in aqueous solution and the ineffectiveness of the hard capsule

It has been postulated that the absorption of vitamin B_{12} takes place at two sites one of which is the stomach and the second in the intestines Available data also indicate that the stomach may secrete substances which may even interfere with the absorption of vitamin B_{12} in the intestine This hypothesis is supported by the findings of Johnson and Berger (1958) who reported that absorption of vitamin B_{12} *can be improved if this vitamin is encapsulated in the* Type III spansule which remains intact in the stomach, but bursts in the intestines Our own studies demonstrated that vitamin B_{12} adsorbed to a resin[2] which possesses the property of retaining this nutrient in an acid pH and of releasing it only at neutral or alkaline pH can be absorbed to a greater extent than the cyanocobalamin This increase in absorption was demonstrated by comparing the urinary excretion of radioactive vitamin B_{12} by subjects receiving ^{60}cobalt labelled vitamin B_{12} adsorbed or not adsorbed to the resin In study I two groups of subjects were used one of which received 50 mcg of radioactive cyanocobalamin in an aqueous solution and the other the same amount of radioactive vitamin B_{12} adsorbed to the resin The radioactivity in the 24-hour urine was almost twice in the second group as compared to the first group being 540 ± 48 and 1140 ± 56 mmcg respectively In study II three groups of healthy individuals were used Groups A and B received orally 50 and 250 mcg radioactive vitamin B_{12} in aqueous solutions respectively The third group (C) received 50 mcg radioactive vitamin B_{12} previously adsorbed to the resin Our results show that subjects in group C excreted again about twice as much radioactivity as those in group A receiving the same amount

[1] Smith, Kline and French s Liptrol

[2] The material was generously supplied as a finely divided powder by Chas Pfizer Co Inc It consisted of an adsorbate of cobalt-60 cyanocobalamin on the carboxylic acid cation exchange resin Amberlite XE-64

of vitamin B_{12} and approximately the same amount of radioactivity as in group B who received five times as much vitamin B_{12} The mean values of excreted urinary radioactivity are 540 for group A 1200 for group B and 1240 mmcg for group C These studies therefore demonstrate definitely that vitamin B_{12} adsorbed to such a resin is more effectively absorbed than that given in aqueous solution

3 Hormonal Influence of Vitamin B_{12} Absorption and Excretion

The utilization and action of vitamins depend to a very large extent on the endocrine activity of the test animals *It is therefore of interest to study the* possible interrelationship between B_{12} absorption and utilization and endocrine functions The effects of ACTH or cortisone on B_{12} excretion and the effect of thyroid on B_{12} absorption will now be reviewed

(a) *Effect of ACTH and Cortisone*

The administration of either ACTH or cortisone will bring about (Meites Feng and Wilwerth 1957) a marked increase in the radioactivity of B_{12} in the urine of rats previously injected with B_{12}^* These data suggest the destruction of the vitamin B_{12} binding substance in the tissues or a cleavage in chemical union between this substance and vitamin B_{12} as a result of the administration of the hormone Another effect of cortisone therapy to rats is the elevation of the serum level of B_{12} (Yamamoto Okuda and Chow 1957) presumably due to the release of B_{12} from the liver *following the destruction of its* B_{12} *binding* substances or simple bond breaking

(b) *Effect of Thyroid*

Thyroidectomy of rats prevents the absorption of orally administered B_{12}^* The absorption can be restored by feeding a diet supplemented with desic cated thyroid or thyroxine (Okuda *et al* 1956) The importance of thyroid activity in the absorption of B_{12} *is demonstrated in the following experiment* Fifty mmcg of B_{12}^* were given orally to test animals and faecal radioactivity was determined It was found that thyroidectomy resulted in a decrease of absorption from 25 5 ± 1 0 mmcg (before thyroidectomy) to 6 2 ± 1 1 mmcg (after thyroidectomy) When the thyroidectomized rats were subsequently given a stock diet containing 0 1 per cent desiccated thyroid for 3 weeks the absorption was increased to normal levels (27 7 ± 1 3 mmcg) This increase in absorption was also reflected in the greater content of radioactivity in various organs The radioactivity in the livers and kidneys was 7 0 ± 0 2 mmcg and 4 7 ± 0 3 mmcg for the thyroid treated rats and 3 4 ± 0 4 mmcg and 3 2 ± 0 4 mmcg for the thyroidectomized rats respectively It is obvious then that thyroid activity plays an important role for the absorption of this vitamin

4 Effect of Pyridoxine Deficiency on Absorption of Vitamin B_{12}

The effect of pyridoxine deficiency on absorption of vitamin B_{12} (Hsu and Chow 1957) was studied with adult male and female rats Half of the animals were fed a pyridoxine deficient diet for 10 weeks and the other half were given the same diet but 1 0 mg pyridoxine daily by injection All animals were given an oral dose of radioactive vitamin B_{12} The radio-activity *in the faecal matter of the pyridoxine treated* rats was consistently and significantly lower than that of the deficient rats while the urinary excretion of the treated animals was higher than that of the deficient animals The radioactivity in the target organs such as liver and kidneys was higher in the treated animals These data taken as a whole suggest an impairment of vitamin B_{12} absorption related to pyridoxine defi ciency which can be reversed by subsequent pyridoxine *administration*

D Agents Enhancing the Absorption of Vitamin B_{12}

It has been pointed out before that the absorption of orally administered vitamin B_{12} is poor by healthy individuals and is almost nil by patients with perni cious anaemia or subjects with total gastrectomy unless co administered with intrinsic factor The mechanism of action is not well understood In addi tion to intrinsic factor preparations other simpler compounds which aid the absorption of vitamin B_{12} by subjects without pernicious anaemia have been reported Such studies will now be reviewed briefly However in order to evaluate the data critically it is important to mention briefly at this point the signi ficance of the methods of estimating absorption

1 Methods for Measuring Vitamin B_{12} Absorp tion

The absorption of orally administered radioactive vitamin B_{12} labelled with ^{60}Co or ^{58}Co can be mea sured by the urinary (Schilling 1953) or faecal excretion (Heinle *et al* 1952) tests or by the hepatic uptake tests (Glass Gellin and Stephanson 1954) Absorption may be estimated from radiometric measurements of faeces scintillation counting of liver projections or determination of urinary radioactivity estimated after injection of a *massive dose of non* radioactive vitamin B_{12} All these procedures involve the use of radioactive material In view of a general and understandable hesitancy on the part of investi gators to allow the test subjects particularly infants

and pregnant women to be exposed to the hazards of radioactivity the oral tolerance test (Castro Lang and Chow 1954) for estimating absorption is often preferred under such conditions It involves either the oral administration of a single dose of 1 000 mcg or a daily dose of physiological magnitude for a longer period of time Increase in serum vitamin levels can be estimated by a microbiologic assay and is taken as an index of absorption When a single dose is given the test involves the drawing of blood 1–3 hours after administration When a small daily dose is used blood specimens are collected over a longer period of time often in weeks or months depending on the efficacy of medication The vitamin B_{12} content in the serum is then determined and compared with that found before treatment The measurement of increase in serum levels may be the physiologically significant procedure However the radiometric methods are more quantitative

2 Intrinsic Factor Preparations

The co-administration of vitamin B_{12} and intrinsic factor preparations by mouth to pernicious anaemia patients in relapse can cause haematological and neurological remission Its primary function is believed to be one of aiding absorption of vitamin B_{12} This property has been demonstrated radiometrically by the urinary or faecal excretion test or by the hepatic uptake measurement test It should be pointed out that the above three tests involve the single administration of the intrinsic factor and vitamin B_{12} On the other hand a prolonged daily intake of certain preparations of intrinsic factor has refractory effects This phenomenon was first demonstrated in clinically healthy subjects by Chow *et al* (1956) as well as Tauber *et al* (1957) They reported that a continual daily intake of certain preparations of intrinsic factor to healthy old subjects for a period of several months may result in a decrease in the vitamin B_{12} serum levels whereas comparable subjects receiving the same amount of vitamin B_{12} daily without the intrinsic factor showed a slight increase over the same period Similar refractoriness was observed (Heinrich 1954 Killander 1958 Lowenstein *et al* 1957 Schwartz, Lons and Meulengracht 1957 Taylor and Morton 1958) with continued intake of certain intrinsic factor preparations by pernicious anaemia patients who originally responding to this therapy went into relapse after a period of several months of daily treatment The decrease in vitamin B_{12} serum levels upon the continuous usage of such an intrinsic factor preparation may be due to the presence of inhibitory substances or some alergic reaction On the other hand not all preparations show the refractoriness As a matter of fact some other preparations can actually aid the absorption of vitamin B_{12} resulting in an elevation of vitamin B_{12} serum levels after several months of daily intake Thus it may be summarized that intrinsic factor preparations may be classified under two categories upon prolonged use—(1) with inhibitory effect and (2) with non-inhibitory effect. The refractoriness of certain inhibitory intrinsic factor preparations is not limited to clinically healthy subjects but also to pernicious anaemia patients so as to cause relapse Tauber *et al* (1957) concluded that the decrease in serum levels and absorption of vitamin B_{12} brought about by the administration of inhibitory intrinsic factor preparations may be a clinically harmful phenomenon and the administration of intrinsic factor unless it is non-inhibitory to pernicious anaemia subjects for a long period of time is not to be recommended and would be ill-advised This belief is supported further by their studies in rats in which it was shown that the lowering of vitamin B_{12} serum levels due to the feeding of inhibitory intrinsic factor was accompanied by signs of B_{12} deficiency of biochemical and clinical nature

3 Enhancement of Absorption of Vitamin B_{12} by D-Sorbitol

Intrinsic factor concentrate has long been known to be the only substance to increase the absorption of vitamin B_{12} from the gastrointestinal tract Recently it has been reported that a substance or substances in an experimental lipotropic elixir (Chow Horonick and Okuda 1956*a*) enhanced vitamin B_{12} absorption in man and in rats and raised serum vitamin B_{12} levels markedly higher than could be expected from an oral preparation containing no intrinsic factor concentrates The active ingredient in this mixture was established to be a crystalline hexahydric alcohol D-sorbitol commonly used as a moisture stabilizer The increase of absorption of vitamin B_{12} has been demonstrated by increase in the urinary excretion of orally administered radioactive vitamin B_{12} Chow Meier and Free (1958*a*) demonstrated that the co-administration of B_{12} and D-sorbitol could also increase the B_{12} serum level at a rate faster than in comparable subjects receiving vitamin B_{12} alone This agent is effective in the enhancement of absorption of vitamin B_{12} by young and old healthy individuals ranging from 15 to 90 years of age It is likewise effective when tested in pregnant women (Chow *et al* 1958*b*) Urinary excretion tests failed to demonstrate the ability of this compound to improve the absorption of vitamin B_{12} by pernicious anaemia patients in relapse or in remission Hence D-sorbitol cannot be considered as intrinsic factor since it is effective only in non-pernicious anaemia subjects

III VITAMIN B_{12} IN HEALTH AND DISEASE

The essentiality of vitamin B_{12} for health and for the treatment of various deficiency diseases has been recognized and demonstrated beyond any reasonable doubt Thus a complete dietary deprivation of this vitamin as in vegans (Wokes Badenoch and Sinclair 1955) the derangement of the absorption mechanism for B_{12} as in Addisonian pernicious anaemia subjects or in completely gastrectomized subjects (Halsted Briggs and Gasster 1957) or the depletion of exogenous B_{12} (Paulson and Harvey 1954) or tissue reserve by parasites (*Diphyllobothrium latum*) (von Bonsdorff 1957) or micro-organisms (diverticuli) (Krevans Conley and Sachs 1954) will in time lead to the development of haematological and neurological symptoms of pernicious anaemia—a manifestation of severe B_{12} deficiency in man—which can be effectively treated with vitamin B_{12} The necessity for receiving an adequate supply of this vitamin is no longer challenged However the practical questions are—firstly whether healthy individuals whose daily diet includes a generous supply of milk and meat require additional exogenous vitamin B_{12} and secondly whether vitamin B_{12} is useful in other situations of mild B_{12} deficiency Unfortunately the available data do not permit us to provide a complete and unequivocal answer to these questions At best these data are only of biochemical level and the physiological significance requires further study The type of studies carried out in this area is exemplified in the investigations among aged and pregnant women

A Vitamin B_{12} Deficiency in the Aged

It has been postulated that marginal vitamin B_{12} deficiencies exist in many aged patients This hypothesis is substantiated by the following experimental findings

Vitamin B_{12} Tolerance Test

By means of the B_{12} tolerance test (Watkin *et al* 1953) it has been demonstrated that old subjects retain more of an injected dose of vitamin B_{12} than do younger subjects The difference in excretion is explicable on the basis that the tissues of the older subjects contain less vitamin B_{12} than do those of younger ones therefore the older subjects retain more of the injected dose

2 Vitamin B_{12} Serum Level and Age wise Regression

If this were true then one might expect the vitamin B_{12} reserves of the aged to be more depleted than those of young persons That such is the case is suggested by the finding that the serum levels of vitamin B_{12} decrease with advancing age (Boger *et al* 1955 Chow *et al* 1956*b* Gaffney *et al* 1957 Tauber *et al* 1957)

3 Achlorhydria and B_{12} Absorption

Glass (1956) has shown by means of his hepatic uptake technique that the absorption of orally administered vitamin B_{12} is poorer in subjects with achlorhydria Similar findings from urinary excretion tests were reported by McIntyre *et al* (1956) Since achlorhydria and hypochlorhydria are common in the aged it is probable that vitamin B_{12} deficiency from poor absorption is not infrequent in the aged

4 Biochemical Evidence

It has been demonstrated that two of the biochemical consequences of vitamin B_{12} deficiencies are decrease in GSH content in the erythrocytes and the increase in the ratio of oxidized to reduced DPN It was therefore of interest to ascertain (*a*) whether the GSH content in the red blood cells of old subjects is lower than that of the young individuals and to compare the vitamin B_{12} serum levels (*b*) to determine the ratio of DPN in the liver of young and old rats The study on GSH was conducted by Tauber *et al* (1957) and their results indicate that the average GSH content of the erythrocytes of the older people is markedly lower than that of young subjects whose vitamin B_{12} serum level was almost twice that of the old Chang *et al* (1958) demonstrated that like vitamin B_{12} deficient rats the ratio of oxidized to reduced DPN in the liver of old rats is significantly higher than that of the adult rats These data therefore indicate possible vitamin B_{12} deficiency among the aged

In searching for an explanation for the lower vitamin B_{12} serum levels in the aged a number of workers have investigated vitamin B_{12} absorption in different age groups by various methods such as the hepatic uptake and faecal and urinary excretion tests However no age wise regression was noted

Tauber *et al* (1957) used histamine diphosphate in both old and young subjects in an attempt to increase the secretion of the gastric juice and simulate the stimulus and stress of digestion Simultaneously a test dose of 2 mcg of radioactive B_{12} was given orally Their results in the urinary excretion tests on young and old subjects both with and without histamine stimulation demonstrated that the urinary excretion of radioactive B_{12} was increased after histamine stimulation in young persons whereas similar treatment in the old subjects resulted in a slight decrease in the urinary excretion

B Vitamin B_{12} in Pregnancy

Vitamin B_{12} is one of the essential factors in human nutrition and in the maintenance of normal health A wide variety of experimental animals such as cattle, swine dogs rats mice and chickens when placed on a low B_{12} diet, develop deficiencies in their reproductive processes and severe growth deficiency in their young Degenerative changes have been observed in the heart liver spleen, thyroid and kidneys of embryos from experimental animals deficient in B_{12} These abnormal changes in reproductive function may be prevented by supplementing the deficient maternal diet with vitamin B_{12}

Experiments with female rats show that a vitamin B_{12} deficiency causes them to have progressively severe reproductive abnormalities with successive litters Third litter young that are born are so debilitated as a result of prenatal injury that they do not survive even when nursed by normal females on stock diet Lepkovsky *et al* (1951) report that vitamin B_{12} administered parenterally at birth enables many but not all of these third litters to survive Rats that were weaned on vitamin B_{12}-deficient diets showed a period of high mortality two or three weeks after weaning generally dying with a severe leukopenia In general it was found that while lactation was impaired by a vitamin B_{12} deficiency ability to produce live young was even more impaired only five live litters resulting from nineteen positive matings for a third litter

In recent years there has been intensified research on the special nutritional requirements of humans under stress The reoriented nutritional programmes in the U S A place considerable emphasis on clinical research aimed primarily at borderline deficiencies

Some of the studies demonstrate a definite connection between the maternal diet and the development of the foetus while others equally well designed and conducted fail to show such an interrelationship In some of the studies of pregnant women the differences between test and control groups are probably so slight that the significance of maternal diet in reproduction cannot be clearly established but in severely depleted populations the importance of the maternal diet may become manifest

In animals it can be demonstrated that under both experimental and natural conditions all degrees of reproductive failure can result from restriction of the maternal diet Sterility resorption or abortion of the foetus the birth of dead or unviable young, and congenital malformations can be induced by dietary deprivation of the pregnant female But even in such simple experiments it will be found that the results depend upon the species examined the diet prior to starvation, the time and duration of depletion, and the evaluation of the offspring Moreover it should be emphasized that in the experiments no attempts have been made to simulate human dietary conditions and it is not likely that in civilized countries under normal circumstances dietary deficiencies are encountered which are analogous to those created in the laboratory

It is not surprising, therefore that available data concerning vitamin B_{12} deficiencies in humans fail to show the dramatic effects upon reproductive processes observed in experimental animals Foetuses have great affinity for this vitamin and therefore, draw from the mother during the gestational period a sufficient supply for their needs during the early months of extra uterine growth Moreover, the adequacy of vitamin B_{12} in the diet of infants in this country is maintained by the liberal use of milk a rich source of vitamin B_{12} Although vitamin B_{12} may be essential for the growth of children its stimulatory effect cannot be demonstrated easily in clinically healthy full term or premature infants but can manifest itself among undernourished children. Difficulties in conducting studies on infants from countries where milk is not consumed and where the diet is poor in vitamin B_{12} lie in the fact that the infants are deficient in other vitamins as well In spite of these difficulties abundant data suggest that a deficiency of vitamin B_{12} has a deleterious effect on growth Ling and Chow (1954) presented data to support the view that this vitamin directly or indirectly plays a part in the accumulation and transformation of glycogen and fat The effect on carbohydrate metabolism is mediated through the synthesis of glutathione

The necessity of receiving an adequate supply of vitamin B_{12} in the diet is no longer challenged If the growing foetus extracts from its host certain concentrations of essential vitamins and B_{12} is known to be one of these numerous questions naturally arise concerning the requirements of B_{12} during pregnancy Does normal pregnancy increase the requirements for B_{12}? If so will a "good prenatal diet supply a sufficient quantity? If the supply is not sufficient what harm if any might result to mother or foetus? Should we give B_{12} supplements to all pregnant women and if so in what quantity? Answers to these and other queries must be tentative ones because of some obvious limitations of our present knowledge However considerable data have accrued which suggest the essentiality of this nutrient in pregnancy and in the newborn, and these data are summarized in the following sections of this communication.

The B_{12} serum level of vitamins may be considered as an index of tissue saturation although the reverse may not be true It has been shown without a doubt that vitamin B_{12} serum levels decline during pregnancy (Heinrich, 1954 Boger *et al* 1956 Okuda *et al*.,

1956*a*) and that serum concentrations progressively fall to levels well below those observed in non pregnant women as pregnancy advances This apparent maternal deficiency of vitamin B_{12} results in a decrease in the GSH content in the erythrocytes Measurement of GSH content (Chow *et al* 1958*b*) in the erythrocytes of pregnant women at delivery shows a content considerably lower than that of non pregnant women or of the foetus Since the decline in GSH is related to B_{12} deficiency one should expect a rise in GSH content in the cells of pregnant women with the administration of vitamin B_{12} before pregnancy This was found to be the case It may be argued therefore that the foetus has a parasitic action for vitamin B_{12} and shows a great sufficiency of this nutrient Under these circumstances it may be expected that the GSH content in the foetal erythrocytes and the vitamin B_{12} foetal serum level should be markedly higher than that of maternal origin That has been shown to be true likewise experimentally

On the basis of the data presented above it appears that vitamin B_{12} is an important factor in pregnancy and lactation of lower animals and may be an important nutrient for pregnant women The latter conclusion is largely inferential from existing data namely that vitamin B_{12} is drawn from the mother to the foetus and creates a marginal deficiency in mothers as indicated by a decrease in serum level of vitamin B_{12} and a decrease in the GSH content in the erythrocytes The parasitic action of the foetus on the mother s B_{12} reserve is compensated at least in part by the increased rate of absorption of vitamin B_{12} during pregnancy (Hellegers *et al* 1957) however the foetus is the main beneficiary

Although it can be demonstrated that the dietary deprivation of vitamin B_{12} in experimental animals is capable of inducing all degrees of reproductive failure human nutritional studies are met with vicissitudes not encountered in the laboratory Under natural conditions the nutrition of the embryo may be disturbed in many ways other than by dietary deficiencies Endocrine defect faulty implantation of the ovum diseases of the placenta interruption of umbilical cord blood flow and maternal metabolic diseases could lead to nutritional disorders of the embryo and foetus It is clear that these nutritional injuries cannot be prevented by improvement of the maternal diet Moreover the embryo may not always be a successful parasite and under adverse environmental conditions may be injured severely while the mother s health is not seriously impaired Sublethal injuries of the embryo or foetus may be the result of borderline deficiencies not at all harmful to the mother It is impossible to predict at the present time what influence low serum B_{12} levels of the mother may have upon her foetus though we know that lowered stores of B_{12} seldom produce clinical manifestations in the mother In spite of very low maternal serum B_{12} levels, the foetus can still show adequate, or even high, serum B_{12} levels

The relationship of B_{12} serum levels and foetal and newborn disease is largely speculative at present however certain recent data suggest that abnormally low vitamin B_{12} serum levels can be found in the blood of newborns with cretinism Allusion has been made in a previous communication to a foetus in which neither the maternal nor cord blood showed an appreciable serum B_{12} level Later the infant was admitted to the Pediatric Service of the Johns Hopkins Hospital with a classical picture of cretinism Since then a second case of athyroid cretinism has been admitted to the Johns Hopkins Hospital in which the same phenomenon was observed At birth this infant had no appreciable serum vitamin B_{12} and none was detected in the mother s blood at delivery Further studies carried out upon cretins admitted to the Harriet Lane Home for Children showed that vitamin B_{12} levels returned rapidly to normal when these infants were treated with thyroid In the case referred to above the mother s serum B_{12} level returned to normal by the sixth post partum week without thyroid or B_{12} therapy The infant s serum B_{12} level did not return to normal levels until after thyroid therapy had been initiated but the rise was rapid thereafter

Since the occurrence of very low maternal serum B_{12} levels is reported in the presence of normal or high foetal serum B_{12} levels and since the low foetal serum B_{12} levels were obtained only in the infants which developed clinical evidence of cretinism the present authors suggest that the foetal serum B_{12} level rather than the maternal is of significance as a finding in cretinism All the above findings would lead one to believe that the unborn hypo- or athyroid foetus shows increased utilization or destruction of vitamin B_{12} to the extent of depleting the maternal stores The stores of B_{12} are rapidly restored to normal once the parasitic action of the foetus is removed after delivery The relationship between thyroid function and B_{12} absorption in the earlier stages of life is as yet imperfectly understood but it is of interest to contemplate the possibility that a critical foetal level of vitamin B_{12} is necessary for the proper development of certain foetal organs There is need for further work in this area of the problem Since thyroid dysfunction is not an infrequent finding in certain mothers who have recurrent late abortions it seems logical to make elaborate studies of B_{12} stores in these patients

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7

Nutritional Factors in Anaemia

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Contents

I INTRODUCTION

With regard to the colouring matter—O this was said to belong to globules in the blood These globules are so minute that I cannot believe any reports of them when they are said to be examined by the common microscope (Abernethy 1826) It is doubtful whether any clinician at the present time would speak in such casual terms of the erythrocyte which is now recognized to be a complex biochemical system appreciation of the full story of which is only now commencing Details of the complex structure and metabolism of the erythrocyte (Ramsey and Warren 1930 Koizumi 1954 Ponder 1954 Prankerd 1955 Behrendt 1957) and of red cell ghosts (Lionetti 1955) opens up an interesting field for speculation as to the complete role of this cell in the body economy Undoubtedly a number of its metabolic processes are concerned with the maintenance of the erythrocyte itself and their breakdown accounts for changes which take place during storage outside the body (Gabrio Donohue and Finch 1955) However no one has shown that the primary function of the red blood cell is other than that elucidated by Lower in 1669 (Foster 1881) namely the carriage of oxygen We do not pay for the air we breathe and perhaps for this reason often forget that oxygen is an essential tissue nutrient This aspect of nutrition is somewhat neglected and will be referred to in the subsequent discussion

II FUNDAMENTAL CONSIDERATIONS

It may appear that undue space is devoted in this review to fundamental considerations and definitions However there is no need to apologize because there exists a good deal of controversy and confusion about the subject of nutrition and anaemia This might be cleared up if more attention were given to these basic issues Much of the evidence quoted in this paper should be accepted only with the provisos implicit in the following discussion

A The Definition of Nutrition

The quality and quantity of the diet are of great importance and defects at this level lead to primary malnutrition (Platt 1954–5) The quality of the diet is determined by the presence of many different constituents in such forms and relative proportions as to allow efficient utilization It should include at most only a minimum of injurious materials However the definition of nutrition is much wider than that of the diet alone (Platt 1954–5 Anon 1957*b*) and may well be summarized by quoting from McCollum Orent Keiles and Day (1939) who state But the field of modern nutrition covers more than qualitative aspects In addition it is concerned with the determination of the requirements for all nutrients and factors which affect their utilization Moreover it involves the study of interconversion and intermediary metabolism of foodstuffs the chemical reactions concerned with the exchange of energy and the economical production of nutrients in available form for man and animals The ultimate end of a series of processes commencing with the growth of food is the delivery to the living tissue cell of all materials which it needs in order to function in a normal way It is in this context that the problem of nutrition and anaemia must be examined

B The Definition of Anaemia

Anaemia is generally understood to mean a condition of deficiency in the amount of haemoglobin in a unit volume of circulating blood with a consequent decreased ability to transport oxygen It might be concluded therefore that anaemia could readily be assessed in terms of haemoglobin levels However there are serious objections to this There is for example the difficulty of fixing a normal haemoglobin level and this will be dealt with later The concentration of cells and haemoglobin in the blood may be affected not only by changes in plasma volume (PV) but also in red cell volume (RCV) The extent to which the former affect haemoglobin levels may not always be appreciated but they can be of practical importance Wilson and Boyle (1952) showed that anaemia or polycythaemia may be simulated by haemodilution or haemoconcentration For example one of their cases with a haemoglobin concentration of 10 g per 100 ml had as much as 30 ml of red cells per kg body weight an amount in excess of the average for normal women (Wadsworth 1954*a*) A pregnant female in Wilson and Boyle s series with a haemoglobin level of only 6 6 g per 100 ml also had 30 ml red cells per kg body weight A male patient with a haemoglobin concentration of 14 4 g per 100 ml had 155 ml red cells per kg body weight! Six patients with a relatively small plasma volume had been diagnosed and treated for polycythaemia by phlebotomy Wahlund (1954) also found that in polycythaemia there was for the same packed red cell volume (PCV) a considerable dispersion of total haemoglobin per kg body weight In a series of ten normal and ten subjects with rheumatoid arthritis Dixon Ramcharan and Ropes (1955) found PCVs of 42 per cent and 36 3 per cent respectively

and that apparent anaemia was due to a 20 per cent increase in plasma volume Differences in RCV between the two groups was not statistically significant An increase in plasma volume from 663 ml to 853 ml during treatment of a child with the nephrotic syndrome resulted in a fall of the PCV from 40 per cent to 31 4 per cent (Barnett *et al* 1951) During active illness in children Macaulay (1954) found a significant increase in plasma volume The effect of diarrhoea in causing haemoconcentration was shown by De Bose and Mondal (1955) In their series normal subjects had a haemoglobin concentration of 95 3 per cent those with non-cholera diarrhoea 109 7 per cent and those with cholera 111 7 per cent Both in health and disease haemodilution or haemoconcentration may cause important changes in haemoglobin levels In true anaemia there may either be relative increase in plasma volume so exaggerating the anaemia (Strauss and Fox 1940 Allington and Taylor 1955) or haemoconcentration which might mask it (Sharpey Schaeffer 1944)

It is important to emphasize that haemodilution may have physiological significance both in health and disease It has been known for a long time that the blood is diluted during pregnancy (Willcocks, 1881 Miller, Keith and Rowntree 1915) This is also true of the growth period and has recently been illustrated in the case of rats (Belcher and Harriss 1957) It may even occur on change of posture (Walters 1934 Widdowson and McCance 1950) In cardiac failure there is also well marked haemodilution (Gibson and Evans 1937 Samet *et al* 1957) One important physiological effect of this is related to blood viscosity The relative viscosity of the blood at the twenty second to twenty-eighth week of pregnancy is 3 84 compared to a value of 4 61 in non pregnant women (Hamilton 1950) This change is of importance in reducing the work of the heart which has to deal with a greatly increased volume of blood More information is required about the interrelation of cardiac work blood dilution and tissue metabolic needs in various circumstances Some aspects of this in pregnancy have been discussed by Hytten and Duncan (1956) but there are other states especially during growth and ageing which need exploration With old age haemoglobin levels fall (Hobson and Blackburn 1953) and this may be a response to lowered metabolic needs and may reduce the work of the heart. What is not known is how far such changes are abnormal and whether physiological haemodilution may be counteracted by administration of haematinics, especially iron A positive response might be incorrectly used as proof of dietary deficiency and might also result in the production of adverse haemodynamic conditions. It has been demonstrated that iron can raise haemoglobin levels in pregnancy (Labate 1939 Jennison and Ellis 1954) and it has been recom mended that iron should be given as a routine a antenatal clinics (Hamilton and Payling Wrigh 1942 Holly 1955) However in spite of the consider able volume of work *already done in this field mu* more study is required especially *to show* whethe true iron deficiency exists and whether there is reduction in the amount of total *haemoglobin* Simpson (1954) demonstrated that many cases o anaemia in pregnancy were not *iron deficient* bu Edgar and Rice (1956) showed that the opposite wa true in their cases and Scott (1954) found that megalo blastic anaemia of pregnancy could masquerade as a microcytic hypochromic anaemia until iron wa administered A similar effect was found by Tasker Richardson and Llewellyn Jones (1956) Berlin *et al* (1953) Gemzell Robbe and Sjöstrand (1954) and others have shown that RCV is considerably increased in pregnancy in spite of lowered haemoglobin levels, although this is not universal (Verel Bury and Hope 1956) The same problem exists in regard to the relative dilution in children In them also iron can raise haemoglobin levels (Sturgeon 1956*a*) Even in young adults prolonged administration of iron can raise haemoglobin concentration to a certain extent (Garry *et al* 1954)

Changes in plasma volume from time to time and less probably changes in RCV bring about variations in haemoglobin concentration which sometimes suggests rhythmical change and may be one manifestation of a more general biological rhythm (Harker 1958) However in individual cases sudden changes in haemoglobin concentration may be quite unpredictable Within a certain range PCV and RCV are closely correlated (Mollison Veall and Cutbush 1950) and the sex difference in PCV is a direct reflection of a difference in RCV per unit body weight between men and women (Wadsworth 1954*a*) Wiklander (1957) also found that in anaemia blood volume remained constant because reduction in RCV was balanced by corresponding increase in PV so that haemoglobin levels would thus reflect true changes in RCV Never theless these conclusions must be regarded as generalizations It is in the individual case that departures from the rule become important and it must be stressed that it is with the individual case that the nutritionist as any other clinician is mostly concerned. It is common practice in nutrition surveys to separate individuals who have haemoglobin levels above a certain arbitrary value at the time of examination, from those who have values below this In this way an assessment of incidence of anaemia may be made Individual variability may have no apparent effect in such a procedure when relatively crude methods for haemoglobin concentration are used but may have when more precise methods are employed. Another

circumstance of even greater importance is that in which the progress of an individual is being followed in response to some therapeutic measure pharmacological trial or physiological event There is now a good deal of evidence about these variations in haemoglobin levels Ward (1904) found a fluctuation of the red cell count of 5 per cent throughout the day Rabinovitch (1923) a change of up to 26 per cent between morning and evening samples McCarthy (1943) on measuring oxygen capacity found a maximum difference between repeated samples during the day of 12 per cent haemoglobin concentration (Haldane scale) Renbourn (1947) showed a statistically significant day to-day variation in PCV and haemoglobin concentration in six subjects and that changes of up to 10 per cent could occur in a single individual s red cell count within a few hours In an analysis of the results of a group of skilled observers, Biggs and Macmillan (1948) showed that differences of less than 14 4 per cent (Haldane scale) were not statistically significant in reflecting a true change in haemoglobin level In a later investigation using more precise methods Biggs and Allington (1951) found a total variation of 6 01 per cent for capillary blood and 5 83 per cent for venous blood of individual subjects and that these variations were in excess of those attributable to the technical error Analyses of individual variability have also been made by Cotter Lancaster and Walsh (1953) in relation to the effects of menstruation and by Wadsworth (1955) who showed their importance in measuring normal recovery from haemorrhage Differences in group mean haemoglobin levels are more likely to be due to technical factors because individual variability would tend to cancel out The increasing use of methods embodying the photometric principle (Kennedy 1926) and the wide distribution of standard blood samples by the Medical Research Council in England and by the National Research Council in America are important advances in epidemiological studies In visual colorimetric methods one factor of possible importance and which may be overlooked is the influence of light intensity (D Silva and Stammers 1945) and this provides a possible explanation for the unexplained discrepancy in haemoglobin levels found by Price Jones (1931) between English and American subjects It is useful to know that the measurement of haemoglobin as oxyhaemoglobin (Bell Chambers and Waddell 1945) is satisfactory under tropical conditions (Wadsworth and Lee 1955) and that there is usually no need to use special reagents (Lehmann and Baird 1949 Cheek 1950)

An idea of the importance of day to day variability in haemoglobin concentration is given by the results of an investigation on goats (Singleton and Wadsworth in preparation) examples of which are shown in the following graphs (Figs 7 1 and 7 2) It should be noted that there was a considerable fall in haemoglobin concentration *before* the onset of labour that the rise immediately following delivery was only temporary and that tests at less frequent intervals could have given a gross distortion of the true picture Similarly the effects of the hormone injection in the second example could have been interpreted in two exactly opposite ways according to the time of blood sampling

One further factor of importance in haematological evaluation is that of time It is now accepted that the life span of the human erythrocyte in the adult is about 120 days Furthermore the length of time necessary for the development of a new cell in the bone marrow until its release into the blood stream may be several days (Lajtha Oliver and Ellis 1954) and in a case of red cell aplasia (Foy and Kondi 1953) reticulocytes only appeared in the blood stream six days after commencement of treatment Therefore the full effect of replacing a deficient erythropoietic factor may take many weeks It is the experience of clinicians that not only is this so but also that improvement may not always follow a steady course but may pass at one stage through a plateau period It should also be noted in this respect that an immediate reticulocyte response may be due to the release of preformed reticulocytes (Seip 1953) and not to an increase in erythropoietic activity The double peak found in the reticulocyte count after haemorrhage may be a manifestation of this (Wadsworth 1955) In spite of this it is commonly found that clinical investigators change a therapeutic agent unless a definite improvement is shown over a short period of time In clinical investigation of anaemia as in any other scientific investigation it is of fundamental importance to provide adequate controls In this instance it may often mean using a single individual both as a control and experimental subject making due allowance for individual variability time needed for adequate response and the natural history of the expected response In regard to the latter it should be remembered that such factors as increased plasma volume relatively short life span of newly formed cells and redistribution of metabolic material inside the body may each play a part The time factor may also be important in relation to dietary deficiency and the appearance of effects Thus the half life of vitamin B_{12} in the liver is about one year (Schloesser Deshpande and Schilling 1958) it takes several years for anaemia to appear following gastrectomy (Paulson and Harvey 1954) and in pernicious anaemia without treatment 25 per cent of cases do not show relapse until the second year (Schwartz and Legere 1944)

If the fundamental function of haemoglobin is to carry sufficient oxygen to the tissues anaemia should

be defined in these terms and haemoglobin concentration can only be an indirect measure of it The full story includes pulmonary and haemodynamic function and the relative proportion of haemoglobin to metabolically active tissue In normal people the RCV is closely correlated with lean body mass (LBM) (Muldowney 1957) although under abnormal conditions, adaptive mechanisms may be used by the body For example in the anoxic conditions of heart failure there is increased pulmonary ventilation and cardiac output and a more efficient removal of oxygen from condition might be of particular value—body weight is likely to be disturbed by abnormal accumulation of water and by other factors Even in healthy people body weight consisting of such variable proportions of fat water protein and minerals is an unsatisfactory basis for standardization Wiklander (1957) and others have demonstrated the extent of variability of blood volume in relation to body weight and Linden (1955) found that PV was closely correlated with the amount of body fat Therefore the close correlation of RCV to LBM is important Lean body mass is however a

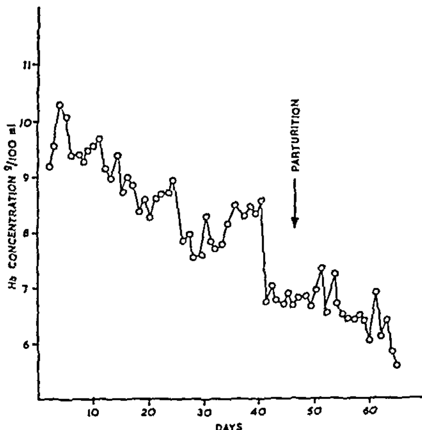


FIG 7 1 DAILY HAEMOGLOBIN CONCENTRATIONS IN A PREGNANT GOAT
(Singleton and Wadsworth in preparation)

the arterial blood by the tissues (Holling 1952) However the production of such compensatory mechanisms should be regarded as an added and undesirable strain on the body tissues

The existence of important individual variability in haemoglobin concentration suggests the desirability of measuring RCV itself in anaemia but if haemoglobin concentrations are variable RCV is more so when one individual is compared with another Up to the present standardization has been attempted by relating RCV to unit body weight Unfortunately in a number of pathological states—and it is in such circumstances that a knowledge of the haemopoietic morphological concept and its needs for oxygen will depend not only on its size but also on its metabolic rate and it has been shown that the thyroid activity influences the normal relationship of RCV to LBM (Muldowney Crooks and Wayne 1957) Blood volume is also affected by muscular activity (Clark and Linden 1948 Kjellberg, Ruhde and Sjostrand, 1949) and Brown *et al* (1954) found in Eskimos that RCV per unit body weight was influenced by basal metabolic rate which changed according to climatic conditions Baugh *et al* (1958) found that the RCV of a group of Eskimos was 32 6 ml per kg and that of a group of Canadians 31 8 ml per kg Sklaroff (1956)

found that there was a reduced blood volume in a group of old people (whose metabolic rate may be presumed to be less than that of younger people), and from his figures it can be calculated that the mean RCV per kg body weight was only 23 8 for both men and women Therefore a functional element should be introduced in the definition of anaemia and this may be provided by the measurement of oxygen consumption Thus the normal state may be defined causal relationship between the two parameters it follows that if the metabolic tissues are depleted because of malnutrition there will also be a depletion of red cell mass even in the presence of adequate haemopoietic nutrients Further, the presence of an inadequate red cell mass might lead to nutritional changes in the tissues because of anoxia The cause of such changes could be obscure unless the relationship of RCV to LBM was known in that instance Finally

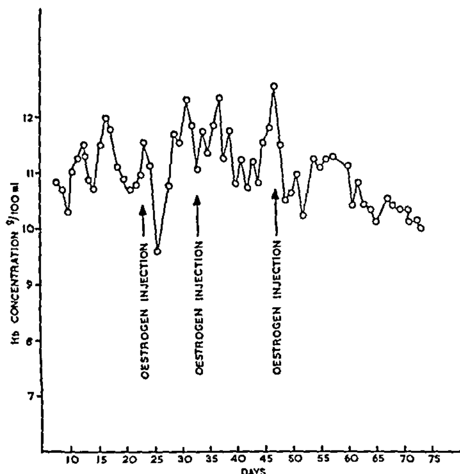


FIG 7 2 DAILY HAEMOGLOBIN CONCENTRATIONS IN A GOAT SUBJECTED TO HORMONE INJECTIONS
(*Singleton and Wadsworth in preparation*)

as that in which the amount of total circulating haemoglobin is sufficient to supply the metabolically active tissue with its particular oxygen needs by means of blood of such viscosity that no undue strain is placed on the cardiac and pulmonary systems Anaemia exists when the haemopoietic tissue is unable to provide an adequate red cell mass to fit these circumstances

The definition of anaemia in terms of LBM and RCV is particularly relevant to the question of nutrition and blood formation For example if there is a tissue depletion and erythropoietic dysfunction might have a common cause In clinical nutrition the problem is the condition of the whole patient and in respect to anaemia this means the simultaneous measurement of the state of the body tissues as well as that of the blood

One main difficulty about sorting out the nutritional factors in anaemia is that natural foods in contrast to laboratory synthetic diets when deficient in one respect are likely to be deficient in others This may lead to argument about specific nutritional

factors concerned in anaemia when these are judged by the clinical effects of particular diets on the body as a whole It is sometimes believed that deficiency of a particular nutrient in a dietary mixture is responsible for the anaemia and another different nutrient for the other signs of malnutrition It is important to consider as already pointed out tissue nutritional defects which are secondary to anaemia and also the relative effects of single nutrient deficiencies on blood and tissues It is commonly taught that in Addisonian pernicious anaemia the patient is well nourished However this may only mean that he is well covered and he may, to reverse a phrase used by Ryan *et al* (1957) be a thin man in a fat man s body ' Vitamin B_{12} is concerned with protein metabolism and mitosis (Rubbo 1953 Johnson, 1958) and in deficiency of this vitamin it would be surprising if tissues other than those concerned with blood formation were not affected It is thus of interest that Read and Asher (1956) found well marked body wasting in pernicious anaemia patients They also refer to the observation of Addison (1855) that the general frame becomes flabby rather than wasted and to those of Jones (1938) 42 of whose 78 patients had lost weight when admitted to hospital It would be of great interest to know more about the body composition in patients with vitamin B_{12} deficiency An instance of similar character is the effect of iron deficiency on the body tissues in view of the implication of this element in tissue enzyme systems Recently it has been shown (Beutler 1957) that there is a measurable deficit in the cytochrome content of animal tissue cells in iron deficiency states This is important in relation to the clinical signs encountered in iron deficiency and usually ascribed to the effects of the anaemia itself

C. The Normal Haemoglobin Level and the Incidence of Anaemia

It follows from the above discussion that no precise single value can be given for a normal haemoglobin level Indeed it was owing to a failure to realize the extent of individual variations in this value that much confusion arose in the field of haemoglobinometry because early standards were based on estimations for only one or two individuals It is now well recognized that 100 per cent had quite different absolute values on different haemoglobinometer scales (Lindsay Rice and Selinger 1926) It is only in recent years that reliable information has been accumulated Because of the great number of surveys that have been made in the past 70 years or so it comes as a surprise to learn how defective is our knowledge about haemoglobin levels of normal people in different circumstances Examination of 121 papers published between 1850 and 1950 involving many thousands of subjects in different parts of the world showed that in only about 24 were data presented in a form which allowed statistical comparisons to be made even fewer were acceptable on technical grounds. When making comparisons it is important to take into account possible climatic effects Relatively sudden changes of temperature affect concentration of haemoglobin and as a rule there is a fall in hot climates and a rise in cold ones (Bazett *et al* 1940 Glaser 1949 Bass and Henschel 1956) In some instances high

TABLE 7 1

Haemoglobin Levels of Presumably Normal Adults

Author	Year	No	Country	Mean Hb level gm/100 ml
Males				
Osgood	1926	137	America	15 8
Jenkins and Don	1933	118	England	15 6
Andresen and Mugrage	1936	40	America (5000 ft)	16 5
McGeorge	1936	50	England	15 2
Sankaran and Rajagopal	1938	125	India	16 6
Myers and Eddy	1939	111	America	15 8
Schepers	1947	100	S Africa (6000 ft)	20 4
Gil and Terán	1948	100	Mexico (7457 ft)	17 7
Berry Cowin and Magee	1952	94	England	15 9
Wadsworth	1954	337	Singapore	16 1
Lancaster and Banks	1957	37	Australia	16 4
Females				
Osgood and Haskins	1927	100	America	13 7
Jenkins and Don	1933	116	England	13 8
Andresen and Mugrage	1936	40	America (5000 ft)	14 5
Sankaran and Rajagopal	1938	62	India	13 7
Myers and Eddy	1939	48	America	13 1
Schepers	1947	50	S Africa (6000 ft)	18 3
Gil and Terán	1948	100	Mexico (7457 ft)	15 2
Hawkins Barsky and Collier	1948	352	Canada	13 8
Berry Cowin and Magee	1952	100	England	14 1
Wadsworth	1954	116	Singapore	13 9
Lancaster and Banks	1957	63	Australia	14 2

environmental temperature leads to haemoconcentration (Wardlaw et al 1935 Kennedy 1939) the picture may be complicated because of seasonal change of diet (Odin 1937) When conditions are stable there is probably an adjustment of haemoglobin levels so that climatic and racial effects are not observed (Wadsworth 1954b) In assessing haemoglobin levels the effects of altitude must be taken into account (Chiodi 1950 Huff et al 1951) Representative values for normal people are given in Table 7 1

Some other results in which there is a departure from the above are given in Table 7 2 Presumably these are indicative of defective nutrition

TABLE 7 2

Haemoglobin Level Lower Than the Expected Normal

Author	Year	No	Country	Mean Hb level gm/100 ml
Males				
Navarro	1937	101	Philippines	14 1
Milam and Meunch	1946	212	America	13 8
Bibile et al	1949	269	Ceylon	13 9
Snell	1950	51	Japan	14 5
Lamprell and Cheek	1952	28	Malaya	11 4
Woodruff and Schofield	1957	100	Gambia	11 8
Roberts and Smith	1957	99	Sudan	14 7
Cotter Kariks and Walsh	1958	691	New Guinea	13 1
Lantz and Wood	1958	79	Mexico	14 5
Females				
Navarro	1937	34	Philippines	12 6
Milam and Meunch	1946	268	America	12 2
Hawkins Leeson and McHenry	1947	196	Canada	12 1
Bibile et al	1949	108	Ceylon	12 9
Lamprell and Cheek	1952	66	Malaya	7 3
Woodruff and Schofield	1957	101	Gambia	10 2
Cotter Kariks and Walsh	1958	669	New Guinea	12 0

It is sometimes suggested (for example—Stewart 1955) that there are different normal standards applicable to people in different communities apparently based on the argument that they display no apparent ill-effects in spite of relatively low haemoglobin levels This is a matter of importance and turns on the question of the ability of the body to adapt The well being also may be more apparent than real Cullumbine (1949) showed that there was a correlation between haemoglobin concentration and muscular function as shown by speed of movement ability to sustain effort and other properties

The effects of a low haemoglobin level may be exerted particularly on the heart (Wintrobe 1946) The body consumption of oxygen may increase 10-20 per cent because of increased work of the heart (Castle 1956) and Irvine (1877) many years ago drew attention to cardiac dilatation in anaemia Paplanus Zbar and Hays (1958) have recently studied this experimentally In a controlled investigation in dogs they showed that total hypertrophy and dilatation of the heart accompanied anaemia Widdowson and McCance (1955) found in anaemic pigs both cardiac hypertrophy and increased cardiac output the output in anaemia was 497 ml per kg per min compared with 167 ml per kg per min in normal animals Scheinberg (1951) was able to measure cerebral blood flow and oxygen consumption in anaemic subjects and found that there was a considerable increase in blood flow a marked reduction in arterio-venous oxygen difference and a diminution in oxygen consumption The latter correlated well with mental status defects Robin and Gardner (1953) also showed that cerebral blood flow in pernicious anaemia was increased in reverse proportion to the RCV It has also been shown that in anaemia renal blood flow is greatly reduced (Bradley and Bradley 1947) There are then a number of important effects on the body caused by anaemia the heart itself may reveal oxygen lack as shown by the occurrence of angina as a symptom of severe anaemia The question must therefore be asked of how far in spite of seeming normal physical ability due to successful adaptation the body reactions account for subsequent ill health and loss of life at comparatively early ages It is not possible to answer this on present evidence but it seems wiser to assume that when haemoglobin levels of any community are lower than those of known healthy people this danger exists

The average haemoglobin level of a community is one measure of the presence and extent of anaemia More precise information is obtained by grouping individuals according to arbitrary levels Further information is obtained from the statistics of treatment centres The introduction of blood transfusion has given another source of information not only of haemoglobin levels of ordinary people but also of potential anaemia only manifest after repeated bleedings (Famulener 1922 Hasebe 1936 Bryce and Jakobowitz 1943) All these sources have their limitations

some of which have already been discussed Statistical analysis of the results of an investigation into the incidence of malnutrition in Uganda (Gongora and McFie 1956) has shown that true incidence was well over twice that given in official reports (McFie personal communication) It is likely that this is true especially for anaemia In England *hypochromic* anaemia is regarded as rare in males but Thomson (1943) reported ten cases in adolescents they had been referred for medical examination by Army Medical Boards and otherwise would not have sought medical advice All had had symptoms for many years and responded well to iron therapy Wallerstein and Hoag (1957) remark on the non specificity of symptoms due to anaemia in children and how diagnosis often only follows observation of pallor on routine medical inspection For these reasons it may be predicted that anaemia already acknowledged to be common is of even higher incidence than is generally recognized

D Erythropoiesis

In understanding the problem of nutrition and anaemia it is necessary to know something about the physiology and chemistry of blood formation because according to definition the final part of the story of nutrition is at the level of cell activity It is currently assumed that all red blood cells arise from the mitotic division of primitive stem cells However whenever a cell divides in this way two daughter cells are formed and contrary to static diagrammatic representation the mother cell no longer exists Thus the problem of blood formation—and its nutrient requirements—must take into consideration not only the division of the stem cells but also the origin and formation of these cells An alternative theory is that erythrocytes are formed by a budding-off process from the parent cell (Bostrom 1948) so that the main problem here would be concerned with the formation of cytoplasmic material rather than with mitotic division and nucleoproteins Duran Jorda (1943) said that red cells arose from the eosinophil granules of leucocytes (Recently Moroshita (1957) claimed that these granules give rise to leucocytes) Again on this theory the problem is one concerned with cytoplasm Yoffey (1954) drew attention to the observation of Damashek and Valentine (1937) that in pernicious anaemia the marrow lymphocytes were more numerous than normal and fell markedly soon after treatment commenced From a number of careful studies Yoffey (1956) has concluded that enormous numbers of lymphocytes disappear from the peripheral circulation into the bone marrow and there give rise to stem cells This is of great interest and has a direct bearing on the ultimate problem of nutrition and haemopoiesis. We may need to turn our attention away from bone marrow to the lymphatic system, especially the spleen The association of the spleen with haematological disorders and the lymphatic tissue with endocrine activity may also be mentioned.

As to the nutrient requirements for blood formation the phase of exploration of dietary needs worked out particularly in the classic experiments of Whipple and his colleagues may now be regarded as virtually complete They have been summarized by Cartwright (1947) who classifies them as vitamins aminoacids and minerals and it would be superfluous to repeat them here Further commentary however, will be made on some items Another phase now rapidly developing is that to do with the proximate requirements at the cell level and the metabolic pathways involved Amongst other things this knowledge brings to light the ultimate reasons why certain nutrients are required in the diet the implication of others previously unsuspected and the predictable results of deficiency A good deal of this knowledge springs from studies on *in vitro* cultures of haemopoietic tissue (Lajtha 1957) and it is necessary to emphasize that this represents only part of the *in vivo* picture In the living body important modifications may arise because of inter tissue competition for nutrients the removal of catabolic products by whatever conditions give rise to stem cells and by factors which influence haemopoietic rate

It is now known that haem synthesis is linked through a succinate glycine metabolic cycle with the Krebs tricarboxylic cycle (Shemin 1955) Descriptions and discussions of haem synthesis have been given by Rimington (1956 1957) and others Rubinstein and Denstedt (1953) found evidence for the presence of the various enzymes of the Krebs cycle in avian erythrocytes Thus this part of the blood formation process is seen to be but one aspect of a more general metabolic chain of events

Through pyruvate the Krebs cycle is linked to the catabolism not only of carbohydrate but to that of fat and protein as well and may be directly influenced by suprarenal cortical activity (Hennes *et al* 1957) Details of globin synthesis are rather less well documented although much of the process seems to take place in the erythrocyte itself and in close association with the synthesis of haem (Thorell 1955 Beutler 1958) and involves the incorporation of amino acids (Kruh and Borsook 1956) The question arises of whether any part of haemoglobin formation involves tissues other than those of the bone marrow and it is interesting to note that purine synthesis can be effected by the liver but not by bone marrow cells *in vitro* (Lajtha 1957) If some essential steps take place in tissues other than bone marrow how far can disease of these tissues affect blood formation? The production of red cells themselves involves all the processes

to do with mitotic division of cells It is in this sphere that the metabolic activities of vitamin B_{12} folic acid and nucleic acids are of great moment (Rubbo 1953 Mueller and Will 1955 Swan Reisner and Silverman 1955) It may be stated that there is no good evidence at present implicating the two vitamins in haem synthesis and Lajtha and Suit (1955) found that haemoglobin formation did not appear to be impaired in megaloblasts The recent successful use in pernicious anaemia of orotic acid (a precursor of pyrimidine) is further evidence of the location of vitamin B_{12} activity (Rundles and Brewer 1958)

III CONSIDERATION OF SOME DIETARY HAEMOPOIETIC FACTORS

A Vitamins

Anaemia has commonly been described in vitamin deficiency diseases in man although it is po sible that the diets which gave rise to such syndromes were also deficient in other nutrients particularly important for blood formation Anaemia has especially been des cribed in beriberi scurvy rickets and pellagra and the numerous vitamins involved in the tricarboxylic cycle and thus in haem synthesis make much of this under standable Evidence for the implication of panto-thenate riboflavin nicotinic acid thiamine and biotin in porphyrin and haem synthesis has been obtained from studies on bacteria (Lascelles 1955) Riboflavin deficiency can give rise to impaired blood formation in animals (Johnstone and Reed 1937 Waisman 1944) and Foy and Kondi (1953) described a case of red cell aplasia which responded to riboflavin This case had no signs of ariboflavinosis and the mean corpuscular haemoglobin concentration (MCHC) was 33 per cent so that the picture looked like an inhibition of cell formation rather than of haem synthesis The absence of other signs of riboflavin deficiency which arise fairly easily with dietary deficiency suggest a local haemopoietic effect These authors drew attention to the anti riboflavin effect of mepacrine a drug which this patient had been taking A case of anaemia due to pyridoxine defic iency has been described in man (Harris *et al* 1956) and sometimes this vitamin has an apparent thera peutic effect in anaemia (Cartwright 1947) In the case described the red cells had a half life of only 20 days and the MCHC was 27 per cent On treatment the serum iron level fell indicating interference with iron utilization in the absence of the vitamin There was also evidence of abnormal tryptophan metabolism which was corrected by pyridoxine As in the case of riboflavin deficiency the rarity of pyridoxine defic iency anaemia and indeed of a pyridoxine deficiency syndrome suggests that the requirements of these vitamins for blood formation must be very small Deficiency of any vitamin involved in the Krebs cycle will of course bring about metabolic change in tissues other than the blood and a complex picture is likely to evolve Thus Ashburn Daft and Faulkner (1947) found that pantothenic acid deficiency caused anaemia in rats but changes were produced at the same time in the suprarenal glands In isolated rat adrenals obtained from pantothenic acid deficient animals steroid production is significantly diminished (Eisenstein 1957) The implication of nicotinic acid in haem synthesis is of interest because anaemia occurs in pellagra although it may be masked by haemo-concentration This vitamin may also be needed for the formation of cortical hormones (Wettstein and Anner 1954) and there is a striking similarity between the clinical picture of pellagra and Addison s disease both show low blood pressure asthenia dehydration pigmentation and diarrhoea Anaemia sometimes severe may be found in Addison s diseae

The rate of haem synthesis from [2 ^{14}C]glycine or [2 ^{14}C]succinate in the red cells obtained from pyridoxine or pantothenic acid deficient ducklings was much lower than from normal cells (Schulman and Richert 1957) The addition of pyridoxine 5 phosphate *in vitro* stimulated the pyridoxine deficient cells to synthesize haem from glycine and succinate Injection of pantothenic acid into pantothenic acid-deficient ducklings one hour before blood was drawn from them restored the ability to incorporate glycine addition of calcium pantothenate *in vitro* was ineffec tive Anaemia from pantothenic acid deficiency has been described in animals (Briggs and Daft 1954)

The anaemia of scurvy may now be supposed to be due to fundamental metabolic derangement rather than to simple loss of blood Ascorbic acid has extremely wide metabolic implications (Meiklejohn 1953) including the utilization of folic acid and vitamin B_{12} (Mueller and Will 1955) In a recent study Cutforth (1958) found anaemia in nine out of eleven patients with scurvy in six the haemoglobin concentration was less than 55 per cent Five had normocytic normochromic anaemia and four had macrocytic anaemia The bone marrow was examined in two cases from each group and all showed normo-blastic hyperplasia Thus in Cutforth s cases the vitamin deficiency did not seem to affect mitotic activity of erythroblasts and it is of interest that ascorbic acid has an important influence *in vitro* on haem synthesis (Goldberg 1958) A different effect of ascorbic acid deficiency was reported by Brown (1955) who showed that it successfully restored megalo-blastic haemopoiesis to normal

B Fat

It is surprising that the close association of fat with haemopoietic material in the bone marrow has not raised more speculation as to whether it plays a part in blood formation In addition to its presence in the marrow, lipid has other associations with haematology It forms an important part of the red cell structure (Ponder, 1954 Turner 1958) lysolecithins are powerful haemolysins and their danger in this respect is only obviated by their prompt removal by the liver when they are metabolically formed in the body plasma lipids play an uncertain part in intravascular coagulation (O Brien 1955 Merskey and Nossel 1957) Erythrocytes are capable of lipid synthesis (James Lovelock and Webb, 1957) and there is active interchange between plasma and red cell cholesterol (London and Schwartz, 1953) A defect in the red cell stroma associated with lipid abnormality has been found by Munn and Crosby (1957) in paroxysmal nocturnal haemoglobinuria Robscheit Robbins and Whipple (1955) found that the concentration of red cell stroma lipids in dogs was increased in anaemia of protein deficiency and haemolysis Bone marrow fat is very labile and is easily depleted after haemorrhage (Evans and Oppenheimer 1955) and in anaemia caused by acetylphenylhydrazine there is preferential removal of linoleic and linolenic acids from the marrow (Evans Baker and Oppenheimer 1955) During fasting in rabbits there is considerable depletion of marrow fat (Evans Riemenschneider and Herb 1954) Evans (1952) showed the presence of esterase activity in rabbit marrow Megel and Gordon (1956) found that this enzyme was influenced by the pituitary gland However, this activity may be concerned more with leucocyte than erythrocyte metabolism (Hardin *et al* 1955)

C. Cobalt

The production of polycythaemia in animals by administration of cobalt was a phenomenon which received much attention formerly but the discovery of cobalamine and that it contained this element seemed to detract from this interest However there is now a return to the idea that cobalt stimulates blood formation apart from vitamin B_{12} Fountain and Dales (1955) successfully treated a case of pure red cell aplasia with cobalt and Thomas (1956) described a case of normoblastic microcytic refractory anaemia which showed a good response to 180 mg of cobaltous chloride daily Schleisner (1956) reviewed the literature on the use of cobalt in anaemia and found in his own cases that cobalt chloride in a dose of 60 mg a day was often effective in anaemia associated with renal disease This author points out that there is an antagonism between cobalt and methionine and cysteine (Orten and Bucciero 1948 Wesley 1951)

Recently Goldwasser *et al* (1958) showed that cobalt chloride resulted in the rapid development of a high titre of an *erythropoietic stimulating factor* (erythropoietin) in rats They further suggest that the site of erythropoietin formation is the kidney (*Jacobson et al* 1957) so that the effect of cobalt in anaemia associated with *kidney disease* (*Gardner 1953* Desforges and Dawson 1958 Erslev 1958) is of great interest

D Copper

There has been relatively little interest in copper and anaemia in humans mainly because it seems that dietary sources are usually adequate Reports about the therapeutic value of copper are controversial. Nevertheless whilst the cause of many cases of anaemia remains obscure it seems worth while to explore further dietary requirements and metabolic fate of copper Butler and Newman (1956) investigated blood concentrations and urinary excretion of copper in normal adults and demonstrated considerable diurnal variations These authors stressed the great technical care needed in such studies and this together with their findings should be borne in mind in the investigation of copper and anaemia

E Protein

Protein forms a major part of the blood and blood forming tissues and haemoglobin forms a major part of the body proteins (Pauling 1955) Moreover 300 mg of globin and 12 5 mg of haem are synthesized each hour (Rimington 1956) and the amount of globin returned to the metabolic pool on catabolism of haemoglobin represents one tenth of the total daily protein requirement (Platt and Wadsworth 1956) Also there is a high incorporation of amino acids into developing red cells (Borsook *et al* 1952 Gavosto and Rechenman 1954) In addition the constitution of haemoglobin and of the numerous enzymes and hormones concerned with erythropoiesis suggests that the requirements for essential amino acids may be critical From this point of view therefore it seems likely that protein malnutrition should readily give rise to anaemia In fact the position is far from clear and will be discussed further

Aschkenasy (1957) found that it was possible although difficult to produce protein deficiency anaemia in rats In these animals the haemoglobin decreased first and the fall was always greater than that of the red cell number The bone marrow showed normoblastic hyperplasia Aschkenasy also found evidence of increased blood destruction and that deprivation of essential amino acids caused a hypochromic anaemia Orten and Orten (1943) found that adequate protein was essential for haemoglobin formation in rats. Miller *et al* (1949) showed that [^{14}C]DL lysine

produced radioactive erythrocytes in the circulation more slowly in protein-deficient dogs than in normal ones Sebrell and McDaniel (1952) found in rats that the relative amount of haemoglobin produced on specific amino acid-deficient diets was fairly closely correlated with that expected from a consideration of the amino acid composition of haemoglobin Nizet and Robscheit Robbins (1950) found that reticulocytes obtained from dogs deprived of protein lacked the ability to mature *in vitro* this was restored by addition of amino acids to the system Rigdon *et al* (1955) found that protein prevented the disappearance of reticulocytes in the blood of ducks following starvation whereas sucrose or vegetable oil was ineffective The complexity of the situation was discussed by Aschkenasy (1957) who pointed out the following complicating factors Foods rich in animal protein also contain vitamin B_{12} so that depletion of such foods might cause effects due to deficiency of the vitamin Protein deficiency causes changes in the gastro intestinal tract which might lead to decreased secretion of intrinsic factor and to interference with absorption Defective intestinal secretion might also lead to modification of the intestinal flora and affect bio synthesis of vitamins Owing to amino acid–vitamin interconversions protein deficiency could increase the requirements for certain vitamins Thus overloading with nicotinamide reduced the anaemia following a protein free diet and haemoglobin formation was greater when vitamin B_{12} was given with methionine than when each was given alone Fox Briggs and Ortiz (1957) found that in chicks methionine and choline had a significant vitamin B_{12} sparing effect and that methionine at a level of 0 15 per cent completely replaced vitamin B_{12} Kratzer and Lantz (1957) found that signs attributable to folic acid deficiency developed in turkeys when they were given an excess of glycine Another important factor is that in protein depletion growth is impaired and thus the usual requirements for haemopoiesis are reduced (Bisson and Wadsworth 1958)

When human subjects lived on a protein depleted diet they showed a loss of metabolically active tissue and also a reduction in the amount of total haemoglobin (Keys *et al* 1950) However the normal ratio of the one to the other was preserved In the production of specific protein depletion in adult man factors such as selective competition for amino acids by parasites or special tissue growth excessive protein catabolism metabolic competition within the body or the effects of antimetabolites may be of greater importance than primary dietary deficiency The effect of the latter is likely to be complicated by the anorexia due to deficiency of essential amino acids (Rose 1957) On theoretical grounds natural dietaries taken in sufficient quantity should provide the protein requirements for tissue maintenance in adults In children a critical factor will be whether the protein deficiency causes cessation of growth For these reasons it is not surprising that there is no straightforward association between clinical syndromes of protein malnutrition and anaemia The situation is made more difficult because nearly all studies have been made on a basis of peripheral blood haemoglobin levels, and this can be seriously affected by changes in plasma volume in such cases Foy and Kondi (1958) did not report the actual dietary intake or precise nutritional status of their subjects Their conclusion that protein deficiency is not an important primary cause of anaemia is therefore open to question

Patients who show serious wasting of body tissues also have depleted amounts of haemoglobin if measurements are made of blood volume Berlin *et al* (1955) measured the blood volume of 66 cancer patients and found that 31 8 per cent were anaemic judged by the RCV in relation to body weight Although haemorrhage and infection complicated a number of these cases anaemia was found in patients without these signs Miller *et al* (1956) found anaemia in 22 out of 38 cancer patients These investigators found evidence of decreased red cell life span thus indicating imperfect red cell formation Furthermore, the anaemia was not related to the presence of local interference because of bone marrow metastases and hypoferraemia with normal iron stores was a common finding indicating a defect in iron utilization rather than iron deficiency Pareira *et al* (1955) demonstrated improvement of anaemia associated with nitrogen retention in cancer patients treated by tube feeding Hollingsworth and Hollingsworth (1955) measured the blood volume in 38 patients suffering from tuberculosis and found an RCV of less than 23 ml per kg in 19 of them In these cases red cell life-span was normal and reduction in plasma volume often obscured the anaemia

Whilst it is true that anaemia is an inconstant finding in kwashiorkor (Trowell Davies and Dean, 1954) this conclusion is based on measurement of haemoglobin levels But anaemia sometimes severe is described in many series Stare and Davidson (1945) remarked that severe forms of anaemia in childhood are almost always accompanied by signs of other deficiencies especially protein The mean haemoglobin level in 48 cases of kwashiorkor investigated by Tovar Escobar and De Majo (1955) was 8 8 g per 100 ml, with a range of 2 8 g to 13 8 g per 100 ml Lantz and Wood (1958) showed that there was a correlation between haemoglobin levels of adolescents and their protein intake Walt Holman and Hendrickse (1956) investigated megaloblastic anaemia in protein-deficient children Of 42 cases admitted during one year, about half had typical signs of kwashiorkor

the adults cells (Hollingsworth 1955) and they also have relatively high mechanical fragility (Yi Yung Hsia *et al* 1954) The increased serum bilirubin in infants is probably a result of a relative liver inadequacy, and not of excessive haemolysis (Billing Cole and Lathe 1954) Therefore the breakdown of red cells cannot be a source of storage iron The importance of this source was supposed to be because of the inadequacy of milk to fulfil the infant s needs Further the picture was based on peripheral blood concentrations which are indeed high at birth (Davidson *et al* 1943, Gottfried Bogin and Levycky 1954 Guest and Brown 1957) and which rapidly diminish during the neonatal period It is during the early months of life that anaemia diagnosed on haemoglobin levels is most common (Wallerstein and Hoag 1957) and this seems to be related to the concentration of iron in the liver (Smith *et al* 1955*a*) When total circulating haemoglobin is measured it is found that there is a steady increase from birth until adolescence (Brines Gibson and Kunkel 1941 Karlberg and Lind 1955) and that the variations during growth in haemoglobin concentration must be due to relative changes in plasma volume The infant s needs for iron must be met partly from iron deposited in the tissues during intra uterine life and partly from the milk ingested however poor this food may seem to be in iron content It has indeed been noticed (Strauss 1933) that sometimes pregnant women with hypochromic anaemia give birth to children who may have anaemia and that this state of affairs may be obviated by giving iron to the mother during pregnancy but this relationship is not always clear (Parish and Brame 1954)

Smith *et al* (1955*b*) conducted an experiment in which normal pregnant women were given repeated small donations of red cells containing radioactive iron Examinations were then made on the infants subsequently born and the relative amounts of iron used for blood formation derived from the mothers and from the post natal diet calculated Unfortunately few details of the infants diet were given If this consisted of the mother s milk it is probable that the iron from that source would be radioactive and of similar specific activity to the iron in the child s body This could explain why there was no relative change in the radioactivity of the child s blood during suckling. If the infants were fed artificially the conclusion reached namely that there was no utilization of dietary iron for haemoglobin formation until 3–4 months after birth is of interest but difficult to understand The inference is that no dietary iron was absorbed or if it was it entered a different metabolic compartment from that involved in haemoglobin turnover Some details of the diet are given in the case of three of the infants studied in two of them this was an artificial feed It is significant that in these two subjects there was a greater proportion of non-radioactive haemoglobin than in the remainder of the series during the first year of life The diet of the other child consisted of the mother s milk and the proportion of radioactive to non radioactive haemoglobin remained more or less constant during the first 200 days of life The child was however also receiving medicinal iron An important conclusion made by Smith *et al* was that the total amount of haemoglobin remained constant until 60 days when a slight increase appeared This did not become appreciable however until 100 days thus implying that whatever iron was absorbed during this time was disposed among body tissues and may have represented a net gain on the part of reserve iron stores

Sturgeon (1956*b*) reviewed the question of iron metabolism in infancy and drew attention to the total iron needs not only for maintenance of the RCV but for growth increases in this the formation of myoglobin and the building up of iron reserves. Sturgeon estimated that the total amount of iron required for increased growth from 8 to 11 months would be about 65 mg or 0 7 mg a day

Some idea of the generally accepted state of affairs may be gained from this brief consideration of the problem There are however two aspects which require elaboration One is the control of iron balance and the extent of iron absorption and this will be discussed in the following paragraphs The other matter is the actual measurement of the amount of iron in the body during growth and its disposal in different organs This has been studied very meagrely but an important investigation was that of McCance and Widdowson (1951) These authors pointed out that Fontes and Thivolle (1925) had shown that the total amount of iron in puppies increased considerably during suckling and they concluded that the source of this iron was the mother s milk McCance and Widdowson showed that in the cat the concentration of iron in the fat free body tissue fell during the first three weeks of life then rose during the next three weeks and was at a still higher level in adult life By contrast there was a progressive fall in the percentage of the total body iron in the blood from birth to adult life During early life there was a substantial change in the relative distribution of iron Thus at birth 22 per cent of the total body iron was in the liver and 24 per cent in the rest of the body tissues apart from the blood At three weeks old there was only 8 per cent in the liver and 48 per cent in the rest of the body It follows from this that studies of iron stores based on liver analysis alone are quite insufficient Furthermore as emphasized by Josephs (1958) many studies of iron absorption are based on iron usage for haemoglobin formation and give no idea of its disposal in the body During active growth it is reasonable to suppose that

much iron is localized at the site of active anabolism but such iron may still be available for haem synthesis

McCance and Widdowson (1951) confirmed that during suckling there is an accumulation of total body iron and that in some species at least all the increments are derived from milk The average newborn infant according to these authors contains 274 mg of iron and at six months by analogy with adult measurements should contain 400 mg Therefore during this suckling period a gain of 126 mg of iron is acquired This amount could be obtained from the ingestion of human milk if 43 5 per cent of the iron was absorbed Feuillen (1954) performed balance studies on 19 infants between the ages of 15 days and 9 months and found an average retention of 53 per cent of the dietary iron intake when this was composed of mixtures of cow s milk In two instances studies were made of infants fed on breast milk one showed a retention of 48 per cent and the other of 78 per cent In a further study Feuillen and Lambrechts (1954) found a mean iron retention of 45 per cent There seems little to justify therefore the recommendation that iron should be given as a routine to all infants (Holt 1955) Milk has often been stigmatized as a poor source of iron and whilst it may be true that this fluid contains a low concentration of the element it is sufficient and presumably right for the physiological circumstances of the infant during the suckling period It may be noted in this respect that the iron content of milk seems to be independent of the mother s dietary intake (Krauss and Washburn 1936) The occurrence of iron-deficiency anaemia in infants must involve other factors and some of these will be discussed subsequently

Many studies of the presence of iron in the tissues have now been made following the demonstration by Rath and Finch (1948) that this could be measured from an examination of bone marrow smears It was to be expected that such reserve iron would be increased in such conditions as haemochromatosis and anaemias not due to iron deficiency and would be depleted in iron deficiency and chronic haemorrhage All these patterns have been in fact demonstrated (Pratt and Johnson 1954 Morse 1955) However, a matter of great interest is the occurrence of anaemia of obscure origin in the presence of adequate iron stores A particular instance of this is the anaemia associated with infection (Stevens Coleman and Finch 1953 Hutchison 1953) There are cases of even greater obscurity (Bjorkman 1956) and sometimes of particular significance there occurs a hypochromic anaemia in the presence of haemochromatosis (Butt *et al* 1956)

There is controversy about the aetiology of haemochromatosis (Anon 1957*a*) particularly as to whether iron deposits are the result or the cause of liver fibrosis The former possibility is of interest in the present instance because it raises the possibility that iron deficiency anaemia may be due to interference with the normal circulation of iron from the point of breakdown of haemoglobin to that at which it is re utilized for haemoglobin synthesis Pinniger and Hutt (1956) drew attention to the early work of Polson (1928 1929 1933) and Cappell (1930) which showed the distribution of iron in the tissues They themselves using parenteral injections of iron found that it appeared in the histiocytes of the spleen lying free in the red pulp and within the Malpighian corpuscles and even in the cells lining the splenic sinusoids and in the trabeculae and capsule Granules were present in the Küpffer cells of the liver and also in the liver cells adjoining the portal tracts Iron was also present in the sinusoidal histiocytes in lymph nodes The amount in bone marrow was less than that in liver and spleen It is of interest that there was a temporary hold up of iron in the capillaries of the lungs for a few hours after injection This phenomenon was also found in man by Andersson (1950) Pinniger and Hutt failed to produce fibrosis in the liver as a consequence of heavy and prolonged doses of iron They point out that in haemochromatosis the iron is inside the epithelial cells compared with its disposition in the reticuloendothelial system (RES) cells in their experiment Kaldor (1954) found that experimental haemolytic anaemia in rats resulted in a marked increase in spleen weight and that this became even more marked after the administration of iron Tissue analysis showed excessive concentration of iron in the liver but much more so in the spleen

Golberg (1957) pointed out that observations in man and animals showed that it is highly unlikely that haemochromatosis could result from severe and protracted haemosiderosis In a recent study Brown *et al* (1957) examined dogs which had been subjected to parenteral iron administration over many years They demonstrated heavy iron deposits particularly in the RES but there was no evidence of fibrotic reaction These authors quote many references to work which showed failure of tissue iron overload to produce cirrhotic changes in the liver They also draw attention to the fact that in published reports there appears to be a complete lack of correlation between the amount of iron and cirrhosis fatty changes in the liver and pancreatic lesions in cases of haemosiderosis Ellis Schulman and Smith (1954) investigated thirteen cases of Cooley s anaemia in which siderosis and cirrhosis were present They noticed that the degree of siderosis bore no relation to the amount of iron given as therapy or by transfusion although exact measurements were not made In one instance however they found that the liver contained 27 36 g

of iron although only 0 5 g had been given From the details in four cases it is seen that although the colour index in one instance was 1 2, that in the others was 0 58, 0 74 and 0 86 respectively

Wyatt Mighton and Moragues (1950) report the case of an old woman who had lived for many years on a very poor diet She received no transfusions, but in spite of severe anaemia the liver, which showed diffuse fibrosis contained 22 5 g of iron In a case reported by Goldish and Aufderheide (1953) a man with a history of alcoholism had chronic hypochromic anaemia in the presence of cirrhosis and haemochromatosis The condition did not respond to folic acid or vitamin B_{12}

In a study of erythrokinetics Spencer Mitchell and King (1957) give details of three cases affected by disease of the lymphoid system In two cases of Hodgkin s disease and one of lymphosarcoma iron disappeared from the plasma about twice as rapidly as is normal All these patients however, were anaemic In a further instance it was shown by external counting of radioactive iron that in lymphosarcoma whilst there was a normal uptake curve for the bone marrow, those for spleen and liver were greatly increased

Experiments of special interest were conducted by Freireich *et al* (1955, 1957*a*) in which they showed that in the presence of an inflammatory process in dogs there was a disorder in ferrokinetics The effect of injection of senescent radioactive cells into the animals on the production of new red cells was measured together with the effect of injections of radioactive transferrin In the presence of inflammation utilization of radioactive iron from senescent red cells was even less in animals with excessive than in animals with normal iron stores The inhibition was related to the inflammatory process because there was a prompt return to normal when this resolved, and a delayed return to normal when the process was prolonged as a result of injections of ACTH Freireich *et al* (1957*b*) also found that in human cases of rheumatoid arthritis iron was taken up by the RES and was not readily released for haemoglobin synthesis.

From a consideration of this evidence it appears that there are circumstances in which iron released from the breakdown of red cells may enter the RES and be trapped there leading to abnormal tissue iron deposits and at the same time allowing insufficient iron to return to the bone marrow for haem synthesis. Such a mechanism offers a possible cause for iron deficiency anaemias which arise in communities where dietary iron intake seems to be adequate if not excessive (Lehmann, 1949*a*) since many of these cases are seen in those areas where cirrhosis of the liver can be expected because of dietary deficiency and where the RES may be affected by parasitic and inflammatory disease

IV THE BALANCE OF NUTRIENTS IN THE BODY

An overall picture of nutrition may be given in diagrammatic form to show the main factors involved (Fig 7 3)

Since the work of Schoenheimer (1942) it is now accepted that the constituents of the body exist in a state of dynamic equilibrium It follows that constant interchange takes place and that there must be a common meeting ground or metabolic pool Radioactive studies have yielded much information about the rate and extent to which different elementary body constituents enter and leave various tissues and how this is modified by circumstances Much of this exchange rate is associated with the rate of degradation and replacement of whole cells (Leblond and Walker 1956) the total exchange rate will depend on the size of the tissue concerned its metabolic rate and on the relative rates of catabolism and anabolism. Some of these factors will be discussed

A The Distribution of Nutrients

Nutrients from the common metabolic pool will be distributed to different compartments according to the factors mentioned In adult life many tissue components exhibit a condition of continuous breakdown and re-synthesis but others (for example collagen) are relatively inert Tissues of high turnover include the liver bone marrow and gastro-intestinal tract, those of slow turnover rate include the skeletal muscles (Benson Kim and Bollman 1955) Thus although striped muscle represents about 30 per cent of the body weight (Mitchell *et al* 1945) it is not a major competitor for some nutrients in the adult body The importance of this concept in nutrition lies in the fact that the balance of activity and thus requirements, can undergo important modification One result of this may be the internal shift of protein from one compartment to another Such shifts are evident from radioactive studies (Yule *et al* 1953) and an interesting example is provided by the work of Cohen (1955) who studied the distribution of protein in the female baboon He found that during the follicular phase of the menstrual cycle a mass of protein approximately equal to the total amount present in the circulating plasma was deposited in perineal tissue With the onset of deturgescence about 30 g of protein was mobilized from the perineum within two to four days. In the case of iron Magnusson Bergstrom and Odeblad (1955) have demonstrated the extent of redistribution in pregnant rats to the foetus placenta, uterus and mammary gland

An important cause of departure from the adult pattern of distribution of available nutrients is excessive anabolism in a tissue The most important examples of this are growth and pregnancy but neoplasms and even functional hypertrophy should also be considered In active growth of tissue endocrine influences are of fundamental importance and Hubble (1957) has discussed the hormone balance which operates in normal growth In connection with nutrition it should be remembered that the endocrine glands themselves may suffer from inadequacy (Samuels 1947 Jacobs 1948 Ershoff 1952 Perloff *et al* 1954) Zubiran and Gomez Mont (1953) demonstrated significant disturbance of endocrine protein deficiency in rats was improved following administration of cobalt As the authors themselves suggest this may have been due to the favourable redistribution of protein to the bone marrow There are conditions in which haemopoietic rate is increased in order to counteract adverse conditions A well known example is the response to anoxia as seen at high altitude (Huff *et al* 1951) In pathological conditions also bone marrow activity may increase Thus in pernicious anaemia there is bone marrow hypertrophy although the products of such increased activity may remain within the bone marrow (Giblett *et al* 1956) In this connection an important parameter is the erythrocyte life span because if for

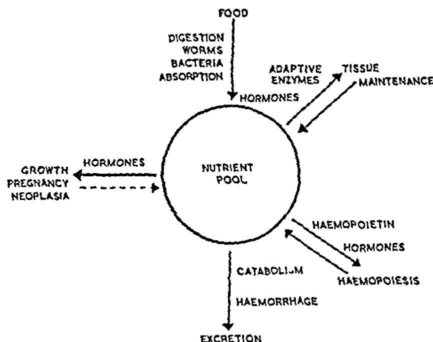


FIG 7 3 SCHEMATIC REPRESENTATION OF THE BALANCE OF NUTRIENTS IN THE BODY WITH AN INDICATION OF SOME POSSIBLE MODIFYING FACTORS

function in 529 adults suffering from chronic malnutrition Haemopoietic activity seems also to be influenced by humoral factors (Carnot and Deflandre 1906 Van Dyke *et al* 1954) which are demonstrable particularly after haemorrhage and also in anoxia and in the presence of anaemia (Borsook *et al* 1954 Hodgson and Toha 1954 Gordon Paleiro and Tannenbaum 1955 Winkert *et al* 1958) Increased activity under these influences will result in an unusual drain of nutrients to the blood forming tissues Thus Fujioka (1956) showed that during recovery from haemorrhage there was accelerated incorporation of iron into erythrocytes Furthermore there was a relative increase of radio iron in bone marrow and a decrease in the liver in anaemic animals Orten and Orten (1945) found that the anaemia associated with example this is halved the rate of production of new cells must be doubled in order to maintain the normal peripheral blood levels For this reason the widespread occurrence of the sickle cell trait (Lehmann 1954) and other abnormal haemoglobin types, is an important consideration in the aetiology of anaemia This adjustment of erythropoiesis rate (Crosby 1957) has given rise to the concept of relative marrow failure (Moore 1957*b*) which is understood from the example already given which demonstrates that erythropoietic requirements will vary inversely with the erythrocyte life span The relative increase in demand for blood forming nutrients will also arise whenever bone marrow activity increases for any other reason The effect of altitude in increasing the demand for blood forming nutrients is illustrated by

the experiments of Criscuolo Clark and Mefford (1955) in which an increase in haemoglobin concentration only followed administration of sufficient iron Another example is given by Whitby (1954) who showed that during recovery from pernicious anaemia the amount of iron in the blood of a patient increased from 434 mg to 1770 mg Thus the very effort at recovery could lead to a state of iron deficiency which did not exist relative to the slow output of cells before treatment By contrast because of reduced erythropoiesis certain nutrients may be present in excess and the amino-aciduria of pernicious anaemia (Keeley and Politzer 1956) may be a manifestation of this Another may be the accumulation of tissue iron in pernicious anaemia and in the anaemia of protein deficiency in animals (Hallgren 1953)

Competition for available protein on the part of different tissues could lead to deficiency for blood formation (Platt and Wadsworth 1956) Hallgren (1953) investigated this problem in rats and was able to demonstrate that under certain conditions of growth and protein shortage anaemia occurred This was mainly shown when total haemoglobin was measured and not haemoglobin levels and there was relative depletion of blood protein in comparison with carcass protein It has often been remarked that anaemia is much more common in artificially fed infants than in breast fed ones Thus Mackay and Goodfellow (1931) found that 42 per cent of infants at 12–13 months old had anaemia when breast fed but 70 per cent when artificially fed This phenomenon was ascribed to defective absorption and utilization of iron in cow s milk but this is not supported by experimental evidence (Josephs 1958) Indeed Feuillen and Plumier (1952) found that infants absorbed more iron from cow s than from human milk Platt (1955) has drawn attention to the increased growth rate of children fed cow s milk and its possible implications in relation to neural myelination Stearns (1939) showed that nitrogen retention was considerably greater in infants fed on cow s milk compared to those on human milk The young infant is able to utilize protein far beyond that which he could possibly obtain from his natural food (Stearns 1956), and therefore a critical factor in growth rate is the amount of protein presented in the diet The difference in growth promotion by cow s milk is emphasized in the case of premature babies In one group there was a gain of 11 7 g per kg a day in infants fed processed human milk compared with 17 3 g for infants fed a cow s skimmed milk mixture (Levine 1956) In the case of anaemia of infants therefore the possibility exists that there is an increased rate of growth and a consequent relative increased distribution of nutrients to tissues other than the blood The analyses of McCance and Widdowson (1951) showed that the body tissues accumulated iron and it may be an unusual increase in this disposition that leads to iron deficiency in the bone marrow

The possibility that iron may be diverted to tissues other than the bone marrow and sequestered thus becoming unavailable for haemoglobin synthesis is another possible cause of iron deficiency anaemia. Part of this problem has already been discussed in relation to hepatic cirrhosis and haemosiderosis Trauma is of frequent occurrence especially in parts of the world where malnutrition exists and it has been shown by Clarke Topley and Flear (1955) that very large volumes of blood may be lost from the circulation without external haemorrhage This blood although undergoing breakdown probably does not release iron for re utilization (Strassman 1954) and may thus be a serious drain from the metabolic pool Infection also blocks the transfer of iron across the placenta and through RES cells (Bothwell Noyes and Finch 1958) Apt Pollycove and Ross (1957) found that in idiopathic pulmonary haemosiderosis there was sequestration of iron into the lung parenchyma in spite of iron deficiency anaemia The latter was corrected by oral iron therapy Nylander (1955) found depression of serum iron and transferrin levels in patients with fractures He thought that this supported the contentions of Cartwright *et al* (1951) who showed that various stresses produced a decrease in serum iron concentration similar to that produced by cortisone and corticotrophin With marked adrenocortical activity there was increased iron storage in the RES and a correspondingly small amount available for haemoglobin synthesis

B Absorption of Nutrients

Even though sufficient nutrients are present in the diet not all of these may be absorbed to an adequate extent The demonstration of Dicke (1950) that wheat protein could produce pathological change in the intestine and lead to malabsorption opened up a new field in nutrition namely the possibility that positive deleterious effects can follow ingestion of normal nutrients This may have wide application in understanding the essential cause of some malnutritional states There are a number of haemopoietic nutrients that may be affected Butterworth Santini and Perez Santiago (1958) found a slow rate of glycine absorption in sprue this was shown also to be the case in pernicious anaemia (Erf and Rhoads 1940) Folic acid is well known to be poorly absorbed in the malabsorption syndrome (Garcia Lopez *et al* 1946 Suarez *et al* 1946) Badenoch and Callender (1954) found that with few exceptions steatorrhoea was associated with poor iron absorption in one case with hypochromic anaemia and poor absorption there was a good response to parenteral iron although in this patient

there was no evidence of steatorrhoea Hawkins Peeney and Cooke (1950) and others have also demonstrated the use of parenteral iron in refractory anaemia and steatorrhoea Edgar and Rice (1956) showed that oral administration of iron prevented a fall in haemoglobin levels in the majority of pregnant women but was ineffective in this respect in 20 out of 89 cases studied

Iron absorption is under intensive study at the present time and the detailed review by Josephs (1958) has already been referred to It seems that the straight forward mucosal block theory (Granick 1948) can no longer be accepted but that iron absorption is a function subject to certain influences Bothwell and Finch (1957*b*) believe that these are fundamentally the state of the iron stores and the rate of erythropoiesis but the effect of reduced haemoglobin levels causing tissue anoxia and affecting iron mobilization from ferritin may be a further factor (Josephs 1958)

Studies of iron absorption by radioactive isotopic and other methods (Steinkamp Dubach and Moore 1955 Josephs 1958) all show that there is a considerable variability both between individuals and between different foods It seems that infants absorb relatively larger proportions of iron in food than do adults Also anaemic subjects absorb more iron than normal subjects (Will and Vilter 1954 Moore 1955) These facts together with the possibility of a control of iron losses undermine the arguments about the causes of iron-deficiency anaemia which are based on the premise that there is a fixed average percentage iron absorption and that iron losses are also fixed It is of interest that Chappelle *et al* (1955) found that total iron losses in mice varied directly with the amount of storage iron

When iron absorption is measured by the amount of radioactive iron which disappears from the intestinal contents it should be remembered that there is a possibility that movements of radioactive ions may not be an accurate measure of translocation of the substrate For example in studies on the movement of ^{32}P into red cells (Wadsworth unpublished observations) it was found that this was a function of the concentration of inorganic phosphate in the suspending medium This is to be expected on physicochemical grounds Assuming as a first approximation that the whole body represents one compartment and the intestinal contents another radioactive ions will move between the two compartments according to the concentration of iron in each

Thus even if there was no actual absorption of substrate iron from the intestinal contents but a high concentration of iron in the tissues radioactive ions would move into the body Therefore interpretation of the results of studies using radioactive isotopes should be cautious unless it is shown that no change has taken place in the specific activity of the intestinal contents during their passage through the canal

It is of practical importance to realize that there is every possibility that iron may accumulate in the body when given in continued doses by mouth (Josephs 1958) and more so after parenteral administration Although it is probable that accumulation of iron does not lead by itself to fibrosis as already discussed it cannot be assumed that it does no harm Thus Golberg and Smith (1958) found in rats that overloading with iron resulted in pathological changes characteristic of vitamin E deficiency In the treatment of anaemia in human subjects careful distinction should therefore be made between iron absorption and iron utilization If the need for iron is based merely on the presence of defective haemoglobin formation and not on evidence of deficient iron stores large and continued doses of iron may be used unnecessarily and have harmful effects (Kaldor 1954) This may be particularly so in the presence of disease affecting the reticulo-endothelial tissues

Until recently it has been supposed that vitamin B_{12} absorption was solely dependent on secretion of adequate amounts of the intrinsic factor although with high dosage this did not seem entirely true The results of experiments with a chemical commercial preparation by Chow Horonick and Okuda (1956) introduced the idea that other substances could lead to vitamin B_{12} absorption Greenberg *et al* (1957) have now shown that certain carbohydrates D-mannitol L sorbose and D xylose and particularly D-sorbitol actively enhance this further evidence of the effect of D sorbitol on vitamin B_{12} absorption was obtained by Chow Meier and Free (1958)

C Excretion

The balance of nutrients in the body may be disturbed by abnormal excretion especially if there is also defective absorption of dietary supply Excessive blood loss, for example is an instance of this A more subtle example is the excessive loss of nitrogenous material which is found in conditions of stress a loss which cannot be balanced by increased absorption This important aspect of metabolic balance was described in relation to trauma by Cuthbertson (1932) who has recently reviewed the problem (1954) Mason (1955) has produced evidence of nitrogen loss following surgical operation in humans and that the adrenal cortex plays a part in these changes This has also been investigated in animals (Long 1956 Munro and Chalmers 1945) The possibility that stress might initiate an untoward nutritional balance and lead to anaemia has been discussed by Platt and Wadsworth (1956) and by Platt (1958) Whether it is an important factor in the aetiology of anaemia in chronic disease is a matter for speculation but Johnston s (1953)

researches on nitrogen balance of tuberculous patients are of interest in this respect It may well be involved also in the haemoglobin shortage in chronic shock Lyons (1943) discussed the need for blood transfusion on the part of patients suffering from chronic shock and the association of body wasting and reduced RCV in chronically ill patients (Lyons and Mayerson 1947) This is a matter of first importance in surgery It was shown by Kehne Hughes and Schlenker (1956) that the operative prognosis in tuberculosis was greatly improved by blood transfusion to correct RCV deficit

D Other Factors

There are other aspects of the problem of balance of nutrients in relation to the aetiology of anaemia but there is only space to say something about two of them namely, the liver and infection

1 The Liver

The liver, as a metabolic competitor with the bone marrow has already been mentioned However this organ is important not only on account of its size and metabolic rate but also because of its position It lies in the immediate pathway of the incoming stream of nutrients absorbed from the gut Van Slyke and Meyer (1913) found for example that during digestion of protein the increased amino nitrogen in the portal blood was largely removed by the liver and that this was associated with an increased amino-acid content of the organ In addition the liver is an important storehouse of iron and of folic acid and vitamin B_{12} (Girdwood 1951 1953) A further factor of possible importance in relation to liver function and its bearing on haemopoiesis is the reduced blood flow through the liver affected by cirrhosis (Bradley Inglefinger and Bradley 1952 Biozzi *et al* 1958) Anaemia has often been described in association with disease of the liver (Krasnow *et al* 1957 Mehrota 1957 Vasavada Arora and Mukerji 1957 Higgins and Stasney 1935 Wintrobe and Shumacker 1933, and others) and the erythrocyte life span may be shortened (Allen Carr and Klotz, 1957) It is generally agreed that such anaemia is macrocytic but with a normoblastic bone marrow thus suggesting the possibility that red cell size is affected by plasma factors It is also commonly claimed that the anaemia can at least in part be explained by an expanded plasma volume Thus Perera (1946) claimed that there was a consistent increase of 10 per cent in this value compared to normal A great difficulty lies in ignorance of the patient s normal blood volume and no measurements seem to have been made of total RCV in relation to his metabolic needs The claim that plasma volume expansion gives rise to a spurious anaemia is not entirely borne out by the evidence Thus if some published values for RCV per kg body weight and PCV are plotted (Fig 7 4) it is seen that in one series there was good agreement A departure from a normal curve may well be explained by differences in technique such as the allowance made for trapped plasma in the haematocrit In another series, an illustration is provided of the great unreliability of judging RCV deficits from estimations of erythrocyte levels

2. Infection and Infestation

A great deal of the anaemia throughout the world occurs in places where infection and infestation are also common and these complications have an important bearing on nutrition (Smith 1955) Unfortunately it is extremely difficult to assess in precise terms the interrelationship between them and tissue nutrition In only one instance indeed does there seem to be a clear causal relationship between an infestation and the production of nutritional deficiency causing anaemia This is in the case of the fish tapeworm which appears to take up appreciable amounts of vitamin B_{12} (von Bonsdorff and Gordin 1952) and apparently there is competition for the vitamin between intrinsic factor and the worm (Brante and Ernberg 1957 1958) It is of interest to note that a haemopoietic substance has been extracted from *Ascaris lumbricoides* (Oliver Gonzalez, 1957)

The well known association between hookworm infestation and anaemia is still a matter of investigation This is mainly because of the difficulty in providing adequate controls Thus Chang *et al* (1949) whilst demonstrating anaemia the degree of which was correlated with the intensity of infestation also showed that non infected people in the same community were also gravely anaemic Likewise Lehmann (1949*b*) found that in healthy subjects the presence of hookworm infection was not associated with anaemia However it is now certain that these parasites bring about blood loss which can be considerable and must be of importance as an aetiological factor in anaemia Roche *et al* (1957) making use of red cells labelled with ^{51}Cr in man found in 21 subjects a blood loss of 2 0 to 251 5 ml daily this was roughly proportional to the worm load In 12 patients with *Necator americanus* infection the blood loss caused each day by a single worm was 3 11×10^{-2} ml $\pm$ 1 73×10^{-2} ml. Loss of blood caused by *Ankylostoma duodenale* was about 0 2 ml for each worm daily Blood lost into the gut may be an available source of iron for absorption (Haliburton 1904) but haemoglobin is digested only to a limited extent (Houston 1955) Brumpt Dansove and Ngeth (1957) found no haematological response in hookworm anaemia to administration of iron by rectum but there was a response when given by mouth.

Much is now known about the composition and metabolic needs of parasites (Fairbairn 1957 von Brand 1957 Daugherty 1957) but until quantitative measures are possible the importance of these cannot be assessed In malaria 75 per cent of the haemoglobin of the host cell is destroyed by the growing parasite (Morrison and Jeskey, 1948) and the malaria pigment found in liver spleen bone marrow and lymph nodes (Clark and Tomlinson 1949) consists of haemin (Rimington 1947) The glycogen stores of the liver become depleted during a *Plasmodium berghei* infection and this is not solely due to anorexia (von Brand 1954) Platt (1957) has drawn attention to the large amount of protein used up during malarial infestation of the monkey and gives a quantitative estimation of this It is also of interest that malarial parasites possess all the enzymes of the Krebs cycle (Speck and Evans 1945 Speck Moulder and Evans 1946) These metabolic activities and disposition of pigment in the RES may thus be important factors in the production of anaemia in malaria

Whilst the occurrence of sickle cell trait may be a disadvantage in some respects it seems to inhibit the multiplication of trophozoites (Allison 1957) an effect also found in foetal haemoglobin carriers The latter might be an important factor in the resistance of infants to malaria

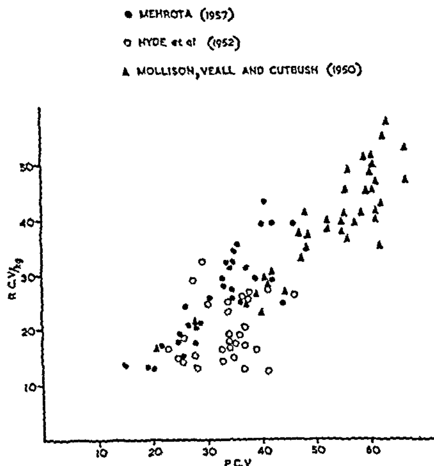


FIG 7 4 THE RELATION OF TOTAL RED CELL VOLUME PER UNIT BODY WEIGHT TO PACKED RED CELL VOLUME IN INFANTS (*Mollison Veall and Cutbush* 1950) AND IN PATIENTS WITH CIRRHOSIS OF THE LIVER (*Hyde et al*, 1952 *Mehrota* 1957)

The question of the importance of the intestinal flora both as a source of the normal requirements of vitamins and as a cause of depletion is still controversial This has recently been reviewed by Gardner (1957) The fact that not all vitamin B_{12} material in faeces is of functional importance is now recognized (Schilling 1954) and much of this vitamin can be recovered from the faeces of germ free chicks (Gardner 1957) One explanation of this is provided by the conclusions of Okuda Grasbeck and Chow (1958) that the bile is an important route of excretion for

vitamin B_{12} In this connection the claim by Foy Kondi and Manson Bahr (1955) that penicillin either orally or parenterally is effective in some cases of megaloblastic anaemia and leads to a rise in plasma vitamin levels is of interest Swendseid Long and Halstead (1957) found that after gastrectomy in the rat anaemia resulted and was associated with decreased liver content of vitamin B_{12} The anaemia responded to aureomycin by mouth but not to oral or parenteral vitamin B_{12} folic acid or hog s stomach The aureomycin did not raise the level of vitamin B_{12} in the liver The interpretation of these results must take into consideration possible metabolic effects of antibiotics Gabuzda *et al* (1958) conducted a detailed experiment on human subjects They found that chlortetracycline (aureomycin) given by mouth resulted in loss of body weight a negative nitrogen balance and a well-marked increase in urinary excretion of riboflavin. In one of four studies made there was also increased excretion of folic acid in the urine There was no definite correlation between the effects of the antibiotic on the bacterial flora and the metabolic effects It should also be remembered that antibiotics especially chloromycetin may depress bone marrow function (Osgood 1953 Erslev 1953, Rigdon Crass and Martin, 1954)

V CONCLUSIONS

There are many complexities in the story of nutrition and anaemia and it is fundamentally important to view the problem in the context of the biology of human life It seems that the stage of first approximations' is no longer adequate and that the artificial conditions of laboratory investigations are of limited value Whilst undue experimentation on patients is to be deplored there comes a stage when some research must be carried out on them That stage seems to have been reached in regard to anaemia The extraordinary frequency with which this sign appears throughout the world and the nature of the problem are such that the subject must be investigated in detail if useful answers are to be found The new fields of inquiry opened up because of availability of modern techniques and the concept of the dynamic state of the tissues of the living organism however do not detract but rather emphasize the importance of knowing just what material is fed into the system from outside

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Neoplasia and Nutrition

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Contents

lobe of the pituitary body would support growth of sarcomatous fibroblasts of the rat or normal chick fibroblasts The normal fibroblast however under went fatty degeneration after a prolonged period of cultivation (Baker and Carrel 1928*b*) When normal and sarcomatous fibroblasts of the rat were cultivated in a mixture of peptones and peptides and amino acids derived from the liver, a temporary proliferation of the normal fibroblast was observed but there was an unlimited proliferation of the sarcomatous fibroblasts (Baker and Carrel 1928*c*) They further noted that the amino acids contributed to the nutrition of the cells but were unable to support life without the addition of peptides and polypeptides The proteolytic products were more toxic to the normal fibroblasts than to sarcoma cultures but the greater acidity produced from the large glycolysis of the sarcomatous cells could account for this difference by altering the rate of reaction of the protein synthesizing enzymes Further Baker (1929) observed that sarcoma cultures grown in a medium containing glycine nucleic acid glutathione haemoglobin, liver ash chick plasma and a peptic digest of casein grew as well as similar cultures when grown in plasma and embryo extract This supplemented medium however was not con ducive to growth of the normal fibroblasts The results of the work of Baker and Carrel clearly suggested that the presence of plasma serum or embryo extract was necessary for continued proliferation One of the beneficial effects of these substances in the medium may be due to the presence of enzymes which slowly degraded proteoses into amino acids (Carrel and Baker 1926 Willmer and Kendal 1932) In view of this work and considering the results of Baker and Ebeling (1939) Vogelaar and Erlichman (1933) and Erlichman (1935) the importance of proteins and their degradation products as well as some unidenti fiable factors in serum and embryo extract in the medium were clearly demonstrated

In 1936 Simms (1936) employed an ox serum ultrafiltrate as a basic constituent in the medium This ultrafiltrate contained no protein (as deter mined by conventional methods) but did represent 0 015M nonprotein nitrogen This ultrafiltrate con tained an A factor which was stable and apparently an acid of small molecular weight and it was reported to be essential for cell maintenance and as a growth stimulant for adult cells *in vitro*

Still later Fischer (1946) published a summary of the work of his group and a considerable portion of this monograph was devoted to the problem of growth stimulants and nitrogen nutrition *in vitro* The growth promoting activity of embryo juice was studied by fractionation procedures and was reported to be associated with labile high molecular weight substances (Fischer 1941*a*) He suggested the activity was related to the nucleoprotein fraction and termed the material embryonin Extraction of lipids increased the activity of this material and while the function of embryonin was unknown Fischer (1946) suggested that it served in a catalytic fashion

Similar results were obtained by Davidson and Waymouth (1945) who pointed out the importance of the nucleoprotein in embryo juice but the growth promoting activity seemed to be associated with the protein *per se* since no correlation was found between the types of nucleic acids present and the growth promoting activity of the extract (Waymouth 1947) It should be pointed out that some of the activity of the embryo extract may be associated with poly saccharides, since mucinase reduced the activity of the embryo juice (Davidson and Waymouth 1943)

Fischer (1941*b*) studied the effects of dialysed plasma serum and embryo extract on the growth of cells and concluded that these dialysable substances were essential for growth irrespective of the presence of embryonin in the medium As a result Fischer and Astrup (1942) attempted to reconstitute the dialysed medium by the addition of amino acids When supple mented with the essential amino acid mixture of Rose (1938) the medium was not so beneficial for cell survival as a supplement based on the analysis of fibrin (Bergmann and Neimann 1936)

This led to some important and interesting conclu sions While both supplements contained all three basic amino acids and tryptophan the fibrin based ' supplement contained aspartic acid glutamic acid proline and cyst(e)ine but no threonine valine isoleucine leucine, nor phenylalanine This led Fischer to the conclusion that cyst(e)ine could not be replaced by methionine and that the concept of e sential and non-essential amino acids for the whole organism did not apply to cells growing in tissue culture (Fischer 1946)

Fischer *et al* (1948) then formulated a medium which in addition to dialysed plasma embryonin glucose some inorganic salts, and the ten essential amino acids contained glutathione glutamine cyst(e)ine mannose galactose fructose diphosphate ascorbic acid some B vitamins and Krebs inter mediates and a number of other compounds known to be of biological significance This medium was designated V 605 and was found to support rapid growth of fibroblasts In a series of growth response studies the supplementary medium was simplified to contain glucose fructose diphosphate glutathione glutamine and the ten essential amino acids except that cyst(e)ine was used in lieu of methionine Using this supplement (V 614) dialysed plasma embryonin and some inorganic salts growth was not appreciably altered from that of V-605 Fischer (1948) then attempted to determine the essentiality of the amino

acids and reported that cyst(e)ine arginine tryptophan glutamine and lysine were the important amino acids for nitrogen nutrition In addition he studied cell response to protein digests and amino acid mixtures (based on the analysis of these proteins) and concluded that tissue cultures may require an unidentified factor of peptide or polypeptide nature

It should be mentioned that while Fischer s V 614 supplement did not contain vitamins or cofactors he did not infer that they were non-essential for growth He interpreted that these substances were probably retained in some combined form in the dialysable plasma and the importance to cell growth of glutamine and fructose diphosphate was demonstrated only because they could be removed by dialysis Consequently they were not the only accessory factors required by the cell (Fischer *et al* 1948)

In 1946 White (1946) reported the maintenance of chick embryo skeletal fibroblasts for a period of 58 days and also reported that he was able to keep heart muscle beating for 54 days on a medium of precisely known constituents The tissues were grown in roller tubes directly on the surface of the glass thereby circumventing the use of plasma embryo extract and/or serum In addition to the ten essential amino acids cyst(e)ine glutathione carotene vitamin A ascorbic acid and nine B vitamins constituted the medium Notably absent from this mixture was glutamine While the medium employed was completely synthetic it should be remembered that Fischer *et al* (1948) pointed out that large amounts of tissue (added to the tubes) could provide the cells with growth promoting substances of unknown composition This was confirmed when Jacoby and Darke (1948) tested White s medium with fowl macrophages grown directly on the glass surface and reported rapid dissemination clumping of cells and cytoplasmic granulation although a few transparent cells remained for a brief period Good growth was obtained when White s synthetic medium was supplemented with 10-20 per cent whole serum

One of the most elaborate media devised was Medium 199 of Morgan Morton and Parker (1950) In addition to a supplement of 21 amino acids (including glutamine) and a comprehensive number of fat and water soluble vitamins Medium 199 included some purines pyrimidines ribose and a variety of intermediates and accessory growth factors The use of Tween 80 in dissolving the fat soluble substances also supplied a source of oleic acid When Medium 199 was used on established cultures and in the absence of serum and embryo extract cell survival was noted for an appreciable period Supplementing the medium with serum did not markedly improve the growth promoting characteristics of the substrate The addition of embryo extract however, resulted in excellent growth

Morgan and his associates modified and improved this medium and designated the new substrate as Medium M 150 (Morgan Campbell and Morton 1955) Using M 150 the survival time of chick heart fibroblasts was the same as for Medium 199 and it also supported equal propagation of influenza and mump viruses in cultures of chorioallantoic membrane (Burr *et al* 1954) From this they developed a depletion technique which could be used to determine the nutritional requirements of the chick embryonic heart fibroblast *in vitro* (Morton Pasieka and Morgan 1956) This will be discussed in more detail in a succeeding section

Medium 199 also has been altered and improved by Parker and his colleagues For example they (Healy Fisher and Parker 1954) were able to get initial cell proliferation of the Strain L mouse cell after which degeneration occurred the cells surviving for almost five months The principal changes in the composition of this substrate were the inclusion of Coenzyme A (Co A) diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) increase in the concentration of cyst(e)ine and glutathione and the deletion of purines pyrimidines and ribose Healy *et al* (1955) also formulated a Medium 858 which again improved cellular proliferation and survival but by supplementing this medium with 20 per cent horse serum again a 20- to 30-fold increase in population of the Strain L cultures was observed Nevertheless Strain L cells could survive and grow at a limited rate in the protein free Medium 858 for an appreciable time

Earle and his colleagues took a somewhat different approach to the problem of a synthetic medium Using the Strain L culture they (Sanford *et al* 1952) tested the growth promoting activity of ultrafiltrates for serum and embryo extract and their respective residues The results indicated that contrary to previous opinion the major growth promoting property of embryo extract was not contained in the high molecular weight residue This enabled them to employ a simplified medium which yielded greater cellular proliferation than controlled cultures grown in whole serum and embryo-extract ultrafiltrate (Sanford *et al* 1953) The test medium in this case was embryo-extract ultrafiltrate horse serum residue and Medium 199 of Morgan Morton and Parker (1950)

Further improvements in growth were obtained with clone 929 (Strain L cells) when an amino acid supplement based on the analysis of horse serum ultrafiltrate (Westfall *et al* 1954) was used This supplement together with niacin *p*-aminobenzoic acid (PABA) niacinamide pyridoxine thiamine Ca

pantothenate, *i* inositol choline riboflavin biotin folic acid ascorbic acid glutathione cyst(e)ine vitamins A, D and E menadione Tween 80 adenosine triphosphate (ATP) were employed as previously reported in Medium 199 (Morgan *et al*, 1950) This supplement rendered the resulting medium superior to unfractionated horse serum (Fioramonti *et al* 1955)

The high molecular weight components of horse serum separated by the Cohn low temperature fractionation procedure (Cohn *et al* 1950) indicated that the gross globulin fraction could be substituted for the horse serum residue (Sanford *et al* 1955) Furthermore removal of the γ globulin from the gross globulin fraction did not appreciably affect the activity Evans *et al* (1956*a*) reported a protein free chemically defined medium which caused definite continued stimulation of clone 929 (Strain L) and was designated as Medium NCTC 107 This medium was improved by the addition of vitamin B_{12} (Evans *et al* 1956*b*), but supplementing with 10 mg per cent of the gross globulins of horse serum substantially increased the growth rate over that obtained in the complete synthetic medium Further improvements included omitting three unsaturated fatty acids and cholesterol and reducing the amount of Tween 80 This new medium NCTC109 (McQuilkin Evans and Earle 1957) was used to establish ten cell lines of clone 929 (Strain L) While these cultures would undergo changes in cell morphology growth rate and population density good growth rates were acquired after the adaptation period

At the time of this writing Strain 2071 of clone L 929 has been continuously cultivated for three years on a protein free chemically defined Medium NCTC 109 The growth rate of Strain 2071 is superior to that obtained using a natural medium but the population density is not so great Nevertheless growth is stable reliable and elegant While admittedly this medium is not perfect it is the first completely synthetic medium reported which can support active rapid growth of a cell strain for an extended period (Earle personal communication)

While the development of a chemically defined medium for continuous cell proliferation of other cells has yet to be accomplished it is obvious that many significant advances have been made toward this end

B Clonal Growth

It is a well-established fact that cells of normal tissue cultivated *in vitro* not only can become malignant but also the sarcoma producing capacities can be markedly different in cell strains derived from a single cell (Earle and Nettleschip 1943 Sanford *et al*, 1950 Goldblatt and Cameron 1953 Sanford Likely and Earle 1954 and Sanford *et al* 1958) Initially the first example of growth of a pure cell strain derived from a single cell was reported by Sanford Earle and Likely (1948) While this method marked a definite forward step in the field of tissue culture it had the disadvantage of being time consuming tedious and could not be readily applied to screening of large numbers of cells

In 1955 Puck and his associates made some important contributions along these lines of endeavour They (Puck and Marcus 1955) developed a method of growing cell colonies from single cells This clonal growth technique permitted screening of large numbers of cells for nutritional studies as well as other purposes Using the HeLa tumour a strain of human epithelial cells from a cervical carcinoma isolated by Gey Coffman and Kubicek (1952) they (Puck Marcus and Cieciura 1956) isolated two cell strains designated as S 1 and S 3 It was noted that the cellular cohesiveness of clone S 3 could be controlled by the source of serum used in the medium If 10 per cent or more human serum was in the substrate the cells grew in a loose highly extended meshwork When bovine porcine or a mixture of equine and bovine sera was used the colonies were dense and compact and the cells were polygonal in shape Cellular measurements indicated that when clone S 3 was grown in human serum the cells had four times the surface area but the same cell volume growth rate and plating efficiency as when grown in the presence of the other sera tested They concluded that human serum contained a factor which could markedly alter the cellular state and that this action of the sera occurred at the cell membrane In later studies (Puck and Fisher 1956) they compared the properties of the S 1 and S 3 clones In the presence of 5 per cent and 10 per cent human serum the plating efficiency of S 1 was approximately nil as compared to 100 per cent for the S 3 clone After nine days of incubation in 20–30 per cent human serum the average colony size was approximately 96 cells per colony for S 1 as compared to 1300 cells per colony for S-3 These were definite examples of differences of cell strains which were derived from the parental HeLa tumour

While initially a feeder layer was necessary for clonal growth (Puck and Marcus 1955) the technique was refined in that the epithelioid cells could be grown in the absence of a feeder layer (Puck *et al* 1956) In studies designed to determine some of the functions of a feeder layer, it was found that the S-3 clone required inositol when grown in the absence of a feeder layer but this requirement was not demonstrable in the presence of a feeder layer of cells (Fisher and Puck 1956) One function of the feeder layer therefore could be assigned to providing growth

promoting substances for the actively proliferating clones

Further refinements of the technique included growing fibroblasts without a feeder layer (Puck Cieciura and Fisher 1957) Embryo extract however had to be present in this substrate Here again were some nutritional differences between epithelial cells and fibroblasts *in vitro* but these examples serve to indicate the complexities encountered in nutritional studies when applied at the cellular level

C. Determination of Essential Components for Growth

During the efforts of many to devise a chemically defined medium little attention was given to a classification of the essentiality of compounds added in the substrates This was understandable since good growth was dependent upon small amounts of serum or embryo extract in the substrate but the resultant media could not be used to identify the small molecular weight growth requirements of the cell Fischer (1948) realized this difficulty and used a chemically defined medium supplemented with dialysed chick plasma serum and embryo extract In this manner he attempted to determine the essential small molecular weight substances for growth He observed a decrease in the radial growth of chick myoblast explants when cyst(e)ine arginine tryptophan glutamine histidine or proline was deleted from the medium but he was unable to demonstrate clearly that these compounds were essential for survival and growth

In 1955 Eagle (1955*b*) devised a medium which could be used to identify the amino acid requirements of the Strain L cells The medium was composed of 19 amino acids a mixture of vitamins and accessory growth factors and 0 5 to 2 per cent dialysed horse serum Under these conditions arginine histidine lysine threonine valine leucine isoleucine cyst(e)ine methionine phenylalanine tyrosine tryptophan and glutamine were shown to be required for survival and growth of the Strain L Only the L isomers of these compounds were active and the non-essential amino acids which were included in the medium were found to be glycine alanine serine aspartic acid glutamic acid proline and hydroxyproline Shortly thereafter he reported that the amino acids essential for the Strain L were also found to be essential for the HeLa tumour (Eagle 1955*c*) This was the first time that the amino acids could be clearly identified as to their growth essentiality *in vitro*

During this time our laboratory was interested in growing the Walker carcinosarcoma 256 in cell suspensions derived from the freshly excised tumour This technique would enable the investigator to study the neoplasm in a condition that may more nearly reflect the nutritional aspects of the tumour *in vivo* and would not permit the tumour to train itself to seemingly artificial conditions encountered in continuous tissue culture This point should be emphasized since Eagle has noted that there was some discrepancy in the essentiality of the aromatic amino acids as a result of continuous culture under tissue culture conditions with the HeLa tumour (Eagle personal communication) This was eventually accomplished by using the amino acid mixture of Eagle (1955*b*) the water soluble vitamins of Medium 199 (Morgan *et al* 1950) and dialysed horse or rat serum It was necessary however to supplement this medium with yeast extract for active cell proliferation (McCoy and Neuman 1956) Fractionation studies of yeast extract revealed that the Walker tumour required asparagine (Neuman and McCoy 1956) When asparagine was included in the medium yeast extract was no longer necessary for good cell proliferation (It was of interest to note that Syverton and McLaren (1957) attributed their success in establishing cell strains from human surgical specimens to the use of yeast extract) With the identification of asparagine it was then possible to determine the amino acid requirements of the Walker tumour *in vitro* These were found to be the same amino acids which previously had been demonstrated essential for the Strain L and HeLa tumours and of course in addition asparagine (McCoy Maxwell and Neuman 1956) While 14 amino acids or amides were shown to be required (when individually omitted from a complete medium) the possibility existed that some of the non-essential amino acids might be necessary for growth if the medium were restricted to contain only the essential amino acids It was found that when the principal source of nitrogen was restricted to only the essential amino acids growth was inferior The addition of alanine proline hydroxyproline aspartic acid or glutamic acid to the restricted medium resulted in no significant increase in growth When however either glycine or serine was added to the medium several fold increase in cell population was observed Thus in addition to amino acids being classified as essential or non-essential for growth and survival it appeared that (at least for the Walker tumour) glycine or serine might best be described as stimulatory amino acids While this concept of stimulatory amino acids was well known in microbiology this was the first time this phenomenon had been demonstrated in the mammalian cell nutrition Since that time it appears that tryptophan may be growth stimulatory for other malignant and normal cells *in vitro* (Eagle Oyama and Levy 1957)

Haff and Swim (1957) have reported the essentiality of serine for the Strain RM3 56 rabbit fibroblasts (however the requirement for serine can be partially replaced by glycine or alanine or several combinations

of the so-called accessory amino acids) and Eagle *et al* (1957) have determined the amino acid requirements for three normal human tissues (liver conjunctiva and intestine) two lines derived from human cancer (KB (nasopharynx) and J 111 (monocytic leukaemia)) They have been shown to require the same amino acids as was previously shown with the Strain L and the HeLa tumour (with the possible exception of tryptophan) Morgan and Morton (1957) determined the amino requirements of chick heart fibroblasts which were found to include the same amino acids as the HeLa and Strain L with the exception of isoleucine and glutamine A résumé of these findings is shown in Table 8 1

Apparently cells cultured *in vitro* although derived from a wide variety of tissues and animal species have similar amino acid requirements (at least as similar as those for the chick rat and man), however there are a number of notable exceptions with respect to asparagine glutamine glycine serine isoleucine and possibly tryptophan This was further exemplified when Eagle *et al* (1957) reported that large amounts of glutamic acid had a glutamine-sparing effect for some of the human cell lines Supplementing with

TABLE 8 1

Comparative Amino Acid Requirements of Malignant and Normal Tissues *in Vitro*

Amino Acid \ Tissue	Malignant						Normal					
	Strain L (Eagle 1955*a* 1955*b*)	HeLa Tumour (Eagle 1955*a* 1955*c*)	Walker Tumour 256 (McCoy *et al* 1956)	Jensen Sarcoma (This lab unpublished)	KB Nasopharynx (Eagle, 1957)	J 111 Monocytic Leukaemia (Eagle 1957)	Chick Heart Fibroblast (Morgan and Morton 1957)	Rabbit Fibroblast (Haff and Swim 1956 1957)	Human Liver (Eagle 1957)	Human Conjunctiva (Eagle 1957)	Human Intestine (Eagle 1957)	Monkey Testicular Tissue (Tytell *et al* 1958)
Arginine	E	E	E	E	E	E	E	E	E	E	E	E
Histidine	E	E	E	E	E	E	E	E	E	E	E	E
Lysine	E	E	E	E	E	E	E	E	E	E	E	E
Glycine	N	N	S	S	N	N	N	N	N	N	N	E
Alanine	N	N	N	N	N	N	I	N	N	N	N	N
Serine	N	N	S	S	N	N	N	E	N	N	N	N
Threonine	E	E	E	E	E	E	E	E	E	E	E	E
Valine	E	E	E	E	E	E	E	E	E	E	E	E
Leucine	E	E	E	E	E	E	E	E	E	E	E	E
Isoleucine	E	E	E	E	E	E	N	E	E	E	E	E
Phenylalanine	E	E	E	E	E	E	E	E	E	E	E	E
Tyrosine	E	E	E	E	E	E	E	E	E	E	E	E
Tryptophan	E	E	E	E	ES	ES	E	E	ES	ES	ES	E
Proline	N	N	N	N	N	N	I	N	N	N	N	N
Hydroxyproline	N	N	N	N	N	N	I	N	N	N	N	N
Cyst(e)ine	E	E	E	E	E	E	E	E	E	E	E	E
Methionine	E	E	E	E	E	E	E	E	E	E	E	E
Aspartic Acid	N	N	N	N	N	N	I	N	N	N	N	N
Glutamic Acid	N	N	N	N	N	N	I	N	N	N	N	N
Asparagine			E	E								
Glutamine	E	E	E	E	E	E	N	E	E	E	E	E

E—essential S—stimulatory N—non-essential I—inhibitory ES—there is still doubt whether this is essential or stimulatory

large amounts of glutamic acid ATP and a source of ammonia as well as high concentrations of aspara gine however could not alter the glutamine require ment for the Walker tumour *in vitro* (Neuman and McCoy 1956)

Thiamine riboflavin pantothenic acid nicotin amide pyridoxal folic acid inositol and choline are the B vitamins essential for some cell lines (Eagle 1955*a* Eagle *et al* 1956) While the Walker tumour has only been shown to require riboflavin thiamine nicotinamide inositol choline and pyridoxal (this laboratory unpublished) the specific requirements for vitamins and cofactors required in catalytic amounts will in all probability not be completely elucidated until the dialysed serum fraction can be deleted from the substrate Some cofactors which have been demonstrated as essential are Co A for the chick heart fibroblast (Morton *et al* 1956) and cholesterol for the HeLa tumour (Sato Fisher and Puck 1957)

In our own laboratories the Walker tumour could be successfully cultivated under clonal growth condi tions in a medium of whole bovine serum and the synthetic components of Medium 2 (Neuman and McCoy 1956) Inocula of 100 cells per Petri dish yielded plating efficiencies of 90-100 per cent When dialysed serum was used a negligible number of colonies developed Inclusion of cholesterol Co A DPN flavin adenine dinucleotide (FAD) and co-carboxylase in the medium failed to improve the plating efficiency This led to a question of other intermediate metabolites and a number of these were tested The addition of pyruvic acid oxalacetic acid and/or α ketoglutaric acid to the medium markedly improved the plating efficiency of the cells while lactic acid *cis* aconitic acid isocitric acid succinic acid and fumaric acid exhibited no physiological activity (Neuman and McCoy 1958) When graded amounts of these compounds were added to the substrate increased cell survival and colony size were noted The optimum concentration was found to be 0 05 mM irrespective of the acid tested Further combinations of these three compounds indicated that the physiological activity was dependent upon the total molar quantity present and was not due to the presence of one component or any combination there of This was an excellent example of how the nutrient requirements of dilute inocula differed from those of massive culture since pyruvic acid oxalacetic acid and α ketoglutaric acid had no effect on cultures with an initial inoculum of 200 000 cells per ml Another facet to be examined is the problem of cyst(e)ine which Eagle demonstrated can be synthesized from a variety of sulphur containing substances in animal cells (Eagle personal communication)

These relations only demonstrate the virtually unlimited field to be exploited in the area of mamma lian cell nutrition From this it appears that the art of tissue culture has developed into a recognized tool which can be utilized fruitfully by the nutritionist

III NUTRITION OF NEOPLASMS *IN VIVO*

The relation of nutrition to neoplasia was usually studied in experimental animals in two ways namely the genesis and the growth of tumours and diets administered to tumour host systems were usually varied in two ways *i e* quantitatively and qualitatively Before considering these aspects however some pertinent factors should be borne in mind—(*a*) a particular dietary regime may not act directly upon the tumour *per se* but only through indirect action (*b*) experimental diets can vary considerably in palatability and therefore affect the total amount of nutrients ingested (*c*) diets composed of highly purified ingredients have a different effect on tumour development from diets composed of natural food stuffs (Silverstone Solomon and Tannenbaum 1952) (*d*) when a diet is deficient in a particular component the effect observed may be due to an imbalanced dietary condition rather than to the specific action of the compound concerned (*e*) obviously the genetic factors cannot be overlooked since the incidence of spontaneous and induced tumours varies markedly between strains of animals (*f*) it is possible that investigations concerned with the use of known car cinogens may represent a special type of carcino-genesis (*g*) while it has been reported that dietary alterations can cause tumour regression (Pearson 1957) it is premature to consider these as a means of preventing cancer in man (Greenstein 1954)

A Genesis of Tumours

1 Effect of Caloric Intake

(a) *Underfeeding*

Moreschi (1909) was one of the first investigators to study the effect of underfeeding in tumour bearing animals and he was given credit by McCay (1947) for extending the work of early British observers which indicated that food restriction slowed or pre vented the growth of tumours in animals Working with a transplanted sarcoma Moreschi (1909) reported that transplantation of tumours was more difficult in the diet restricted mice and that this group lived longer than the controls Further the tumours grew in rough proportion to the amount of food given the animals Rous (1910) conducted similar studies with

rats but he failed to observe any effect by dietary restriction Then followed a period in which no apparent active interest in the relation of nutrition and neoplasia was shown

This interest was revived, however by the classic work of McCay and his associates (McCay, Crowell and Maynard 1935, McCay *et al* 1939 McCay, Sperling and Barnes 1943 McCay 1947, 1952) They reported that chronic dietary restriction (adequate for maintenance but insufficient for growth) would prolong the life of the rat and retard the onset of senescence After a retardation period of 1150 days *the animals still had the ability to mature when given* an adequate diet sufficient for growth Further this moderate degree of underfeeding reduced the incidence of spontaneous tumours (McCay *et al* 1939) Several investigators have reported that underfeeding may partially or wholly inhibit the formation of tumours, as well as exhibit a delaying effect on the time at which the tumour appears This has been shown in spontaneous mammary tumours (Tannenbaum 1940*a* 1947 Visscher *et al* 1942, White *et al* 1944, Rusch Johnson and Kline 1945*b*, Huseby Ball and Visscher 1945) hepatomas (Tannenbaum and Silverstone 1949*d*) leukaemia (Saxton Boon and Furth 1944) and pulmonary tumours (Tannenbaum 1940*a* 1942*a*, Larsen and Heston 1945) It has also been demonstrated in several induced tumours such as skin tumours (Tannenbaum 1940*a* 1942*a*) and sarcomas (Tannenbaum 1942*a* Rusch *et al* 1945*b*)

The effects of overfeeding have not been extensively followed primarily because the condition of forcing an animal to eat is an artifact which may cause many complications too difficult to interpret Further in many experiments the control animals on an unrestricted diet become somewhat obese and the use of *ad libitum* diets for control purposes may in the main be examples of overfeeding Consequently attempts to feed animals *beyond these limits would yield no* pertinent findings

Since chronic underfeeding exerted such a marked effect on the genesis of the tumour some other problems arise concerning the nature of this influence These aspects however should be considered in the light of some closely related studies *i e* experiments where the carbohydrate and/or fat content of the diet was reduced This is especially true since restricting the amount of carbohydrate and/or fat in the diet has affected the genesis of a number of spontaneous and induced neoplasms in a fashion similar to underfeeding (Visscher *et al* 1942 Tannenbaum 1942*a* 1945*a* 1945*b* White *et al* 1944 Rusch *et al* 1945*b*, Rusch Kline and Baumann 1945*a* Tannenbaum and Silverstone 1949*d* Boutwell Brush and Rusch 1949*a*) Thus for the purposes of this discussion the term caloric restriction will include underfeeding as well as the specific restriction of carbohydrate and/or fat from the diet

(b) *Caloric Restriction*

Since the caloric restriction seemed to inhibit tumour formation rather consistently there are a number of factors which can modify the degree of this influence Some of these include—degree of restriction versus degree of carcinogenic stimulus chronic versus intermittent restriction the significance of body weight, time of imposing caloric restriction and some specific effects of restriction

Most of the investigations regarding caloric restriction and tumour genesis used diets reduced from one third to one half that given the controls Greater degrees of restriction were not used primarily because they would not be *adequate for body maintenance* When smaller increments of dietary restrictions were employed however the incidence of tumours appeared dependent upon the extent of the restriction (Boutwell *et al* 1949*a* Tannenbaum 1945*a* 1945*b* Tannenbaum and Silverstone 1949*d*) and included spontaneous mammary carcinomas hepatomas and induced skin tumours As might be expected the carcinogenic stimulus can overcome the caloric effect *if it is sufficiently high* Tannenbaum (1945*a*) however, demonstrated that when the dose of the carcinogen was large enough to produce tumours in practically all the experimental animals increased amounts of caloric restriction produced a corresponding increase in tumour latency These results could be used to explain the somewhat conflicting reports on the relation of caloric restriction and tumour genesis For example King Casas and Visscher (1949) studied adrenal adenomas in *C3H* mice *In the* ovariectomized mice they noticed that caloric restriction did not affect the incidence of adenomas Since all of the ovariectomized mice developed *tumours this* could well be an example of a high carcinogenic stimulus overcoming the caloric restrictive effect as pointed out by Tannenbaum and Silverstone (1953*a*)

(c) *Intermittent Fasting*

Carlson and Hoelzel (1946) investigated the effect of intermittent fasting on prolongation of life span. They used three different fasting treatments *i e* one day in every two three and four days respectively While the life span was prolonged (optimum treatment one fasting period in three days) they did point out that the pre-experimental condition of the rat was an important factor Nevertheless the development of mammary tumours was retarded in *proportion to* the amount of fasting while there was no *drastic* retardation of growth. Tannenbaum and Silverstone (1950) also studied this problem in mice The animals

were fasted for a 24-hour period twice weekly and they reported that this degree of intermittent fasting had no inhibitory effect on the incidence or rate of formation of spontaneous mammary carcinomas nor on their subsequent growth They did point out however that the mice fasted intermittently consumed the same amount of food during the week and grew as well as the control animals This immediately poses the question of the significance of body weight rather than specific dietary regimes

(d) *Body Weight Significance*

Tannenbaum and Silverstone (1949*b*) conducted an interesting series of investigations along these lines Two groups of mice were fed 2 4-dinitrophenol (DNP) or sodium fluoride and a third group was housed at 45–55 F The mice in the cold room and those receiving DNP ate about 10 per cent more food and the sodium fluoride group consumed about 10 per cent less food than did the controls All treatments however resulted in a significant reduction in body weight and incidence of spontaneous mammary carcinomas Under similar experimental conditions the incidence of primary lung adenomas was also inhibited in groups receiving DNP and sodium fluoride and the average body weights of these treatments were less than that of the controls So here is an example in which the metabolic rate was increased (low temperature or DNP) or caloric intake was voluntarily decreased (sodium fluoride) The end result however is an imposed caloric restriction for the requirements of the host These experiments exemplify the intimate relation between caloric intake metabolic growth and the nutritional needs of the animal In an extension of this work Tannenbaum and Silverstone (1949*c*) administered a thyroid extract to mice and noted that 40–45 per cent more food was consumed yet this group weighed about 10 per cent less than control mice While this increased caloric intake did not markedly affect the incidence or time of appearance of induced skin tumours the formation of tumours was more closely related to the average body weight While there are other experimental results which may be considered somewhat conflicting (Tannenbaum and Silverstone 1953*a*) it does appear that the caloric restrictive effect and low body weight may be interdependent

Waxler (1953 1954) and Waxler Tabor and Melcher (1953) obtained some interesting data when obesity was induced experimentally by the administration of gold thioglucose When virgin C3H female mice were given gold thioglucose the ones which became obese consumed more food and the average time of appearance of spontaneous mammary carcinomas was 246 days as compared to 303 days for controls At the same time mice (receiving gold thioglucose) which did not become obese showed the same time of tumour appearance as the untreated mice Similarly mice receiving gold thioglucose but kept at control weight by pair feeding showed the same time of tumour appearance as the control animals One interesting point, however was that if the obese mice were reduced and held at the weights of control animals the time of tumour appearance was even longer than that of the control group In this latter study the average time of appearance of tumours in the obese mice was 219 days in the controls 367 days and 365 days in the previously obese reduced mice (Waxler 1954)

(e) *Time of Imposing Caloric Restriction*

In view of these last results it might be well to consider some of the studies conducted on the relation of the time of instituting caloric restriction and tumour genesis Tannenbaum (1942*a*) noted that tumour formation was significantly inhibited as long as caloric restriction was imposed before the tumours began to appear Further if caloric restriction was instituted on mice (which developed multiple mammary carcinomas) after the first tumour appeared the proportion of animals with multiple tumours was reduced (Tannenbaum 1953) This was exemplified further when combinations of high and low-calorie diets were used during and after a period of administering a carcinogen (Tannenbaum 1944*a*) The experiment was conducted so that one group of mice remained on a high-calorie diet throughout the whole period and another group received a low-calorie diet for the duration of the experiment These were designated as Groups HH and LL respectively A further group (HL) received a high-calorie diet during the period the carcinogen was applied (10 weeks) and were then given a low-calorie diet (52 weeks) Another group (LH) received a low-calorie diet during the period of application of carcinogen and were then placed on a high-calorie diet As might be expected tumour incidence was highest in the HH group and lowest in the LL group The point of interest however, was that the LH group showed a tumour incidence comparable to the HH group and the tumour incidence in the HL group was comparable to the LL group Thus it appeared that caloric restriction inhibited tumour development rather than the initiation stage of carcinogenesis This will be discussed in more detail presently

(f) *Mechanism of Action of Caloric Restriction*

The actual mechanism of the inhibition of tumour genesis by caloric restriction may be several fold since this effect was noted in many different types of tumours When an experimental animal is placed on a dietary regime so that the mean body weight is affected

many other changes occur Not only are tissue and organ weights affected but the over all metabolism is altered and the changes are reflected in levels of fluid constituents organ function and rates of metabolic pathways Since the case of the spontaneous mammary carcinoma may represent a special effect of caloric restriction it will be considered first

Loeb (1921) observed that undernourishment caused a failure in maturation and an occasional regression of follicles in the ovaries of guinea pigs Evans and Bishop (1922) and Asdell and Crowell (1935) noted that under nutrition conditions caused rats to become anoestrus In 1942 Visscher *et al* (1942) reported an irregularity of oestrus when female mice were held on a calorie restricted diet These animals also exhibited a decrease in incidence of spontaneous mammary carcinoma This work was extended by the histological studies of Huseby *et al* (1945) who noted a distinct atrophy in the genitals of calorie restricted female mice While the development of breast tumours may be inhibited by a low calorie intake the underlying mechanism appeared to be associated with decreased ovarian activity in mice which resulted in reducing the production of oestrogen There are several ramifications of these hypotheses namely Trentin and Turner (1941) suggested that partial starvation invoked an additional requirement of oestrogen to produce the equivalent amount of lactogenic hormone necessary for duct growth As a consequence the pituitary is definitely involved This was extended by Mulinos and Pomerantz (1940 1941) who introduced the term pseudo-hypophysectomy to describe results in animals under extended calorie-restricted regimes because of the similarity to hypophysectomized animals Boutwell *et al* (1948) suggested that the increase in size of the adrenals and an apparent increase in glyconeogenesis indicated a relative adrenal hyperfunction which may explain the inhibiting effect of caloric restriction

Before considering other modes of action on tumour genesis, it may be well to consider some hypotheses concerning the process of carcinogenesis This concept was the result of a number of workers but only a few will be mentioned here Berenblum (1941) Berenblum and Shubik (1949) and Rous and co-workers (Rous and Kidd 1941 MacKenzie and Rous, 1941 Friedwald and Rous 1950) utilized specific carcinogens for the induction of skin tumours and they concluded that there were two general processes in carcinogenesis—(*a*) the initiation stage in which the carcinogen produces changes on normal cells which are rendered labile toward tumour formation and (*b*) the developmental stage in which the labile cells are stimulated to produce malignant cells after which growth proceeds As one might anticipate the initiation stage has been shown to consist of more than one step (Shubik 1950 Klein, 1953 1956 Orr 1955 Vesselinovitch and Gilman 1957) and the second stage can be initiated in a number of ways such as further application of the carcinogen the use of a co-carcinogen or combinations of these (Berenblum and Haran 1955) Nevertheless from the extensive work of Tannenbaum (1953) it does appear that the mode of action of caloric restriction in these cases may be closely associated with the developmental stage of the tumour It should be mentioned that Bullough (1950) suggested that the developmental stage of carcinogenesis was influenced by the mean mitotic activity of that particular tissue Since the mitotic activity was definitely inhibited by caloric restriction and a greater inhibition was observed with increasing amounts of restriction he concluded that the unavailability of carbohydrates and/or carbohydrate intermediates for the production of energy necessary for mitosis was responsible for the caloric restrictive effect on tumour genesis

2 Effect of Protein

The effect of dietary protein on the well being of the host as well as tumour genesis assumes a different role from that of calorie-restricted diets While the protein can be oxidized for energy its principal function lies in supplying the animal with essential amino acids for growth As a result the quality of the protein exerts a profound influence on the host When the amount of dietary protein is restricted below certain limits the effect observed may be due to limited amounts of one or more specific amino acids rather than protein *per se* For example casein is known to be low in cyst(e)ine for dietary purposes and similarly gliadin for lysine Other incomplete proteins are deficient in several amino acids and chemical treatment of some complete proteins can render them inadequate for growth Further crude preparations of proteins can contain appreciable amounts of vitamins and cofactors while others contain specific vitamin antagonists, *i e* avidin in egg albumin Therefore it seems more convenient to limit the discussion of this topic to investigations which employed dietary proteins in sufficient amounts to promote growth Other studies will be considered under the topic of amino acids and/or vitamins

Generally speaking dietary protein has little or no effect on tumours arising from any organs or tissues other than the liver When mice were fed diets varying from 9 to 45 per cent casein no differences were noted in the incidence average time of appearance or rate of growth of spontaneous mammary carcinomas (Tannenbaum and Silverstone 1949*a*) neither was the occurrence of metastases or prolongation of life affected after appearance of these spontaneous tumours Tannenbaum and Silverstone (1953*b*) did observe an effect of dietary protein on the incidence

of spontaneous mammary carcinoma when deliberate critical conditions were employed When the diet contained from 10 to 46 per cent casein in one experiment (C3H mice) and from 4 to 30 per cent casein in another experiment (DBA mice) (but the caloric intakes were adjusted to maintain an equivalent body weight) it appeared that decreases in dietary protein hindered the genesis of spontaneous tumours These latter observations however represent a special case Rusch *et al* (1945*a*) noted no effect of varying the dietary protein on the formation of induced sarcomas when the mice were held on a caloric restricted diet Tannenbaum and Silverstone (1949*a*) also reported no effect of dietary protein on formation of induced skin tumours and sarcomas

The relation of dietary protein to the formation of hepatomas presents a somewhat different picture Miller *et al* (1941) observed that an increase of dietary protein caused a retardation of hepatoma formation and Kensler *et al* (1941) indicated that it was one of the factors involved in retarding tumour formation This however has been ascribed to an effect of riboflavin in the diet (Miner *et al* 1943 Rusch *et al* 1945*c*) and the concentration of riboflavin in the liver (Miller *et al* 1948) Tannenbaum and Silverstone (1949*a*) noted that mice receiving diets containing 9 per cent casein had a lower incidence of spontaneous hepatomas than those given 18 per cent or more casein in the diet The differences observed however were apparently not due to the amount of protein *per se* since 9 per cent casein and 9 per cent gelatin diets did not result in a higher incidence of hepatomas than those containing 9 per cent casein (Tannenbaum 1953) Griffin Clayton and Baumann (1949) administered *p*-dimethylaminoazobenzene to rats and noted that more hepatomas appeared when the diet contained 12 per cent casein as compared to 24 per cent casein however with a diet containing 12 per cent casein and sufficient methionine to equal a diet of 24–36 per cent casein the number of hepatomas approximated that containing 24 per cent casein They concluded that both casein and methionine improved the hepatic retention of riboflavin by the rats fed azo dyes and this could account for the difference in tumour formation Tannenbaum (1953) pointed out the fact that increase in dietary protein enhanced the formation of spontaneous benign hepatomas but inhibited the incidence of malignant hepatomas induced by azo dyes From this he concluded that the liver stands out as a unique tissue with regard to the influence of nutrition on tumour formation

3 Effect of Amino Acids

White and Andervont (1943) studied the effects of a low-cyst(e)ine diet on the development of spontaneous mammary carcinoma in C3H female mice No mammary tumours developed in the animals receiving the low-cyst(e)ine diet while mice on a high-cyst(e)ine diet (0 5 per cent added cyst(e)ine) had the same tumour incidence and about the same time of appearance as those on a dog chow diet Since they noted that the mice on the low-cyst(e)ine diet exhibited an irregular oestrus cycle they implanted diethylstilboestrol pellets subcutaneously and raised the tumour incidence on the low-cyst(e)ine diet from zero to approximately 45 per cent From this they concluded that the tumour suppression was due at least in part to a lack of oestrus (White and White 1944*b*)

Larsen and Heston (1945) investigated the effects of a low-cyst(e)ine diet on the incidence of spontaneous pulmonary tumours in mice When the food consumption of the cyst(e)ine supplemented group was restricted to that of the cyst(e)ine deficient treatment no differences in tumour incidence were noted White and White (1946) observed a similar effect on azo dye induced hepatomas except that there was a delay in the time of tumour appearance

Lysine deficient diets reduced spontaneous mammary carcinoma formation from 97 per cent to 25 per cent in virgin female C3H mice (White and White 1944*a*) These results together with those of the low-cyst(e)ine diet effect on mammary tumours (White and White 1946) may be similar to those ascribed to a caloric restricted diet This is especially true when one considers the final body weights of the different experimental groups

White White and Mider (1947) extended their experiments to a study of the effect of cyst(e)ine lysine and tryptophan deficient diets on leukaemia induced by methylcholanthrene The control diets were those using supplements of each respective amino acid While the cyst(e)ine deficient diets (4–5 per cent casein) definitely inhibited the development of leukaemia the lysine deficient (12–14 per cent gliadin) and the tryptophan deficient diet (peroxide-treated casein) did not have a similar effect The tumour latency however was prolonged in the lysine- and tryptophan deficient groups They pointed out that these results were not due to caloric restriction since there was a marked decrease in tumour formation in the cyst(e)ine restricted group but not in the lysine or tryptophan deficient treatments

Dunning and associates (Dunning Curtis and Maun 1950*a* 1950*b* Dunning and Curtis 1954 1955) also examined the role of tryptophan in tumour genesis and noted that the addition of 1 per cent DL tryptophan to the diet enhanced the development of mammary tumours induced by diethylstilboestrol but the addition of 4 per cent tryptophan to the diet inhibited tumour formation This latter result was attributed to the interference of tryptophan with

proper assimilation In studies using 2 acetylaminofluorene in the diet supplements of tryptophan to a casein hydrolysate (tryptophan free) diet increased the incidence of hepatomas and bladder cancers when compared to animals receiving a diet of an equivalent amount of casein One point of interest in these studies is that while the tryptophan supplemented groups did not maintain their body weights they did develop a relatively high incidence of tumours Similar effects on the development of induced multiple mammary tumours and hepatomas were noted when the diet used was synthetic (Dunning and Curtis 1954) The work of Griffin *et al* (1949) concerning methionine in the diet has previously been considered

4 Effect of Fat

A high fat diet generally but not always promotes the development of tumours Watson and Mellanby (1930) were one of the first investigators to study this aspect They observed that diets containing from 12 5 to 25 per cent butter fat caused a marked increase in induced skin tumour formation Following this several investigators examined the fat effect on a variety of tumours and it appeared that most of the tumours were stimulated by high dietary fat but some were not (Baumann Jacobi and Rusch 1939, Baumann and Rusch 1939, Jacobi and Baumann 1940 Lavik and Baumann 1941 1943 Tannenbaum 1942*b* Rusch *et al* 1945*b* Boutwell *et al* 1949*a* Benson Lev and Grand 1956) For example the incidence of spontaneous and induced breast tumours was definitely stimulated by high fat diets (Tannenbaum 1942*b* Dunning *et al* 1949 Silverstone and Tannenbaum 1950) and it does appear that induced skin tumours of mice fed fat-enriched diets have a small but consistent increase in incidence (Tannenbaum, 1942*b*, 1944*b* Lavik and Baumann 1943 Rusch *et al*, 1945*b* Tannenbaum and Silverstone, 1947)

The effect of high fat diet on the formation of hepatomas requires some consideration While there are reports that fat-enriched diets accelerated the formation of hepatomas as compared to diets containing little fat (Opie 1944, Kline *et al*, 1946) there are reports to the contrary (Miller *et al*, 1944*b* Silverstone 1948) Apparently the type of fat incorporated into the diet is an important factor in this respect (Kline *et al* 1946 Miller *et al* 1944*a* 1944*b* Jardetzky, Visscher and King 1955) For example hydrogenated coconut oil definitely inhibits hepatoma formation while olive oil crisco and lard stimulate tumour formation but not so much as an equivalent amount of corn oil

Lavik and Baumann (1941) examined various factors of fat and noted that the tumour promoting activity (at least for induced skin tumours) was in the fatty acid fraction of the lipid Glycerol and the non saponifiable fraction exhibited only slight enhancement of tumour formation but ethyl laurate was as effective as the natural fat in stimulating tumours The tumour promoting activity of the fat could be increased by heating at 300°C for one hour while treatment with ultraviolet light or catalytic oxidation failed to affect the activity of the natural fat

While it appears that the incidence of spontaneous and induced breast and skin tumours is in many cases increased with fat-enriched diets dietary fat apparently does not affect the formation of spontaneous and induced leukaemia (Lawrason and Kirschbaum 1944) pulmonary tumours (Tannenbaum 1942*b*) or induced sarcomas (Baumann *et al* 1939 Tannenbaum 1942*b* Lavik and Baumann 1943, Rusch *et al* 1945*a*)

Some mention should be made with respect to the time of administering high fat diets for stimulating tumour formation Lavik and Baumann (1941) noted that the most effective period of administering the fat-enriched diets was from one and one half to three months after the beginning of the application of the hydrocarbon (the carcinogen being applied for a two-month period) The results of Tannenbaum (1944*b*) support this finding Apparently fat has little or no effect during the initiation stage of carcinogenesis but is much more influential (as in the case of caloric restriction) during the developmental stage of the tumour

5 Effect of Vitamins

Owing to the bio-catalytic action of vitamins it is only reasonable to assume that they could be intimately associated with carcinogenesis and a number of investigators have examined this relationship In this discussion however the fat soluble vitamins will be omitted since there have not been any clear-cut cases demonstrating the relationship between the genesis of neoplasms and these vitamins

Kensler Sugiura and Rhoads (1940) reported that liver coenzyme I and riboflavin were markedly lower in rats fed butter yellow and the incidence of induced hepatomas was greatly reduced when 200 μg of riboflavin per day were included in a diet of casein brown rice and carrots (Kensler *et al* 1941) The importance of riboflavin in inhibiting tumour formation was also pointed out by Miller *et al* (1941) but this protective effect of riboflavin can be masked however by a multiple vitamin deficiency For example Miner *et al* (1943) administered *p*-dimethylaminoazobenzene to rats maintained on a highly purified diet which contained B vitamins in amounts just adequate for maintenance They observed that the incidence of tumours was low in this case On the other hand when large amounts of riboflavin were added to a diet containing 12 per cent casein no hepatomas appeared

In this study the reduction of all of the B vitamins in the diet was sufficient to reduce tumour formation rather than if riboflavin had been reduced singly in which case hepatoma formation would have increased

Griffin and Baumann (1946) examined the effects of different azo dyes upon riboflavin storage in the liver and reported that the more potent carcinogenic dyes caused a greater reduction in hepatic riboflavin At present it is generally accepted that the level of liver riboflavin is the influential factor in affecting azo dye induced tumours rather than the amount administered in the diet (Miller 1947 Kensler 1947 Griffin and Baumann 1948) When the riboflavin content in the liver is high the incidence of azo dye induced tumours is low and vice versa

Kensler (1949) studied the ability of liver slices to destroy N N-dimethyl *p*-aminoazobenzene and reported that livers with a low hepatic riboflavin (induced by feeding low dietary riboflavin or protein or including the dye in the diet) had a decreased ability to destroy the N N-dimethyl *p* aminoazobenzene Decreasing dietary thiamine or choline or the addition of biotin or adenine to the diet had no effect on the liver s ability to destroy the dye Mueller and Miller (1950) in liver homogenate studies identified the cleavage products of 4-dimethylaminoazobenzene as N N dimethyl *p*-phenylenediamine and aniline (neither of which was carcinogenic when tested) They postulated this could account for the protective action of dietary riboflavin against carcinogenesis since riboflavin adenine dinucleotide could activate the liver homogenate system to destroy the carcinogen

The protective effect of riboflavin was investigated on different carcinogens and it appeared that dietary riboflavin had a greater protective action against *p*-dimethylaminoazobenzene than its *o* methyl or *m* methyl derivative (Geise *et al* 1946) Further riboflavin seemed to have little or no effect on the inhibition of hepatomas when rats were fed acetyl aminofluorene (Harris 1947*a*) Weisburger Weisburger and Morris (1954) used [9 ^{14}C]acetylamino fluorene in riboflavin-depleted and supplemented rats They observed that while the absorption of the carcinogen was delayed in the riboflavin depleted group there was no difference in the protein bound radioactivity in the tissues The relation between dietary riboflavin and acetylaminofluorine was extended by Morris Wagner and Velat (1955) and it appeared that while a high riboflavin level in the diet did not affect the incidence of liver tumours there seemed to be an increased incidence of mammary tumours and squamous cell carcinomas of the ear duct Further Morris and associates (Morris and Robertson 1943 Morris 1947) had observed earlier that additions of riboflavin to the diet increased the occurrence of spontaneous mammary carcinomas

Riboflavin deficient diets had no effect on the incidence of intra-cranial tumours although the deficiency did shorten tumour latency (Russell 1945) and Boutwell *et al* (1949*b*) demonstrated that diets low in thiamine and riboflavin had no effect on induced skin tumours From this it is apparent that riboflavin may exert a protective action in the inhibition of hepatomas induced by dimethylaminoazobenzene dyes but it does not necessarily affect the activity of other carcinogens nor the occurrence of other types of tumours

Diets low in pyridoxine have been shown to reduce the takes of the Flexnor–Jobling carcinoma two types of transplanted mouse tumours methylcholanthrene induced skin tumours (Kline *et al* 1943) and dimethylaminoazobezene induced hepatomas (Miller Baumann and Rusch 1945) and biotin deficiency (by the administration of egg albumin containing avidin) retarded the genesis of dimethylaminoazobenzene induced tumours (du Vigneaud *et al* 1942 Burk *et al* 1943 Kline Miller and Rusch 1945 Harris 1947*b*)

Additions of choline to the diet had no effect on the formation of tumours (Miller *et al* 1941 Jacobi and Baumann 1942) although severe choline deficiency in rats has been reported to induce tumours of the liver lung and pancreas sarcomas haemangioma carcinoma of the bladder and cirrhosis (Copeland and Salmon 1946 Engel Copeland and Salmon 1947) Apparently pantothenic acid and thiamine have little or no effect on tumour genesis (Morris and Lippincott 1941 Dobrovolskaia Zavadskaia 1945)

6 Effect of Inorganic Compounds

To date there has been no conclusive evidence that inorganic compounds natural to the diet stimulate or inhibit tumour genesis While there are some reports which describe effects most of these would come under the category of special cases For example King Spain and Clayton (1957) noted that the incidence of azo dye induced tumours was markedly reduced by the inclusion of 300 ppm copper in the diet but they attributed this largely to the catalytic destruction of the dye by the copper for a diet containing physiological amounts of copper had no effect on tumour genesis For a more complete consideration of this aspect the reader is referred to the work of Shear (1933) Stern and Wilheim (1943) and Tannenbaum and Silverstone (1953*c*)

B Growth of Tumours

The term tumour genesis was rather clear cut and required no explanation but the term tumour growth may require some consideration In the case of spontaneous or induced neoplasms growth refers to the enlargement of the tumour after the stages of

carcinogenesis Where transplanted neoplasms are concerned growth does not include the establishment of the tumour implant Only after the tumour has 'taken does growth occur This latter point can be more clearly understood when one considers the interesting observation of Algire and Chalkley (1945) who examined the vascular reactions after implantation of the neoplastic tissue After tumour transplantation capillary proliferation occurred in about three days and growth did not commence until the initial vascularization was complete Devik *et al* (1950) working with implants of the Walker carcinosarcoma 256 reported that the initial inflammatory reaction occurred within 48 hours This was followed by the formation of a connective tissue network which held the neoplastic cells and a rapid ingrowth of capillaries When the capsule was organized the establishment of the tumour was complete and then growth proceeded

As might be expected the effects of a particular dietary regime on the growth of an established tumour has in the main relatively little effect, since the tumour derives its nutrients from the body and seems to be more or less immune to the host s regulatory system Any restrictive diets usually affect the well being of the host more than the neoplasm The end result being that while the growth of the tumour may be affected it still proceeds at the expense of the host This has been exemplified in Mider s concept of the tumour as a nitrogen trap Even when the tumour host system is placed under such drastic conditions as prolonged fasting the established tumour continues to derive its nutrients at the expense of body particularly the carcass (Mider Tesluk and Morton 1948) This was also demonstrated in the radiometric experiments of LePage *et al* (1952)

Some effects on tumour growth have been achieved by the use of specific antagonists Azaserine (an antagonist for glutamine) and the anti folic acid compounds (aminopterin and amethopterin) serve as examples of this type of approach Most of these investigations however, may be more appropriately placed in the realm of experimental chemotherapy so they will not be considered in detail in the present discussion

1 Effect of Caloric Intake

Generally speaking caloric restriction retards tumour growth Moreschi (1909) and Rous (1914) were the first to demonstrate this with transplanted rat and mouse tumours but Rous did point out that the Flexnor–Jobling carcinoma if allowed to grow for a short period was relatively unaffected by a most rigorous underfeeding regime Nevertheless most reports indicated that caloric restriction will reduce the growth rate of tumours This has been demonstrated with rat carcinomas (Sugiura and Benedict, 1926), Sarcoma 180 (Bischoff, Long and Maxwell 1935 Bischoff and Long 1938) mouse mammary carcinomas (Tannenbaum 1940*a*) and transmitted mouse leukaemia (Flory *et al* 1943) With some tumour types (myeloid leukaemia) there was evidence that the life span of the underfed mice was increased over that of control mice but the experimental conditions used could easily have affected the establishment of the leukaemia rather than its growth (Flory *et al* 1943)

It is of interest that obese hyperglycaemic mice and mice with induced alloxan diabetes survived significantly longer than control animals when given intraperitoneal injections of the Ehrlich ascites tumour (Jehl Mayer and McKee 1955) But here again the period of tumour takes" may have been affected Goranson and Tilser (1955) studied the growth of the Novikoff hepatoma and the Walker carcinosarcoma 256 in alloxan-diabetic rats While the intraperitoneal implants of the tumours were affected with respect to tumour take and growth subcutaneous implants were relatively unaffected

Ball Wick and Sanders (1957) examined the influence of some glucose antimetabolites on the growth of the Walker tumour and reported that 2-deoxyglucose significantly retarded the growth rate of the tumour When the administration of 2-deoxyglucose was terminated the growth rate of the Walker tumour approached that of the controls Ingle Altamero and Flores (1956) overfed rats bearing the Walker tumour with a fluid medium carbohydrate diet which was administered by gastric intubation The carcass weight gains in the overfed rats were greater than those of the controls but the growth of the tumours was unaffected In all, it appears that while caloric restriction may reduce the growth rate of some tumours it does not represent a practical means of controlling growth since the well being of the host is also placed in jeopardy

2 Effect of Protein

When protein was included in the diet in amounts sufficient for good growth of the experimental animals variation of the protein content within these limits had no effect on tumour growth This was demonstrated with spontaneous mammary carcinomas and sarcomas (Rusch *et al* 1945*a* Tannenbaum and Silverstone 1949*a*) If the protein content of the diet was restricted to approximately 5–6 per cent, the growth of the tumour was somewhat inhibited but the carcass weight losses of the tumour bearer were appreciable This was observed with the Walker tumour and the Hepatoma 31 (Devik *et al* 1950 Green and Lushbaugh 1949 Voegtlin and Thompson, 1949)

One of the most striking results concerning this protein problem came from the work of Mider and

his associates It is a well known fact that tumour bearing animals when housed together will often consume the partner s tumour This is essentially true if the tumours are implanted subcutaneously and are allowed to ulcerate Further there is no question that tumour bearers exhibit anorexia especially during the phase of the greatly accelerated tumour growth (Mider 1953) With this in mind let us consider the remarkable studies of Richter (1942–43) in which self regulatory functions were examined Self selective dietary experiments conformed with biochemical investigations concerning the altered needs of the st Thus it was not too far fetched that the iorexia of the tumour bearer reflected a change in ietary essentials in order to meet the demands of the imour host system as contrasted to the demands of a normal animal If such a change did occur then it was possible that substance(s) could be stored in excess in the neoplastic cells or destroyed by them

To test this hypothesis Mider and associates used three diets which were isocaloric and contained the same amounts of protein carbohydrate and fat The sources of protein were vitamin free casein lyophylized whole rats and lyophylized whole Walker tumours Rats bearing the Walker tumour and consuming the tumour diet maintained their appetites and carcass weights much better than the tumour bearers on the other two diets The tumour and carcass diets had no appreciable effect on weight increments in the normal animals Tumour bearers receiving the casein diet until they were losing weight from anorexia were shifted to the other two diets The group changed to the carcass diet continued to lose weight and there was still a loss of appetite The rats however which were shifted to the tumour diet resumed eating and began to gain weight Mider (1955) pointed out that the principal net effect of feeding the tumour to its host seemed to be the prevention of carcass weight loss and it was suggestive that cancerous rats have different nutritional requirements from normal animals

This work was extended to compare the effects of feeding dietary tumour on body weight and tumour weight (Millar *et al* 1957) The diet (T 24) containing 24 per cent lyophylized tumour tissue had nearly the equivalent amount of nitrogen as one containing 20 per cent casein (C-20) When the tumour bearers were fed the same amounts of food the rats ingesting the T 24 diet gained more total body weight grew larger tumours and showed less carcass weight loss Examination of excretory products indicated that the animals ingesting the tumour diets excreted less urea nitrogen more allantoin nitrogen and about the same amount of ammonia nitrogen as the casein diet rats (White *et al* 1957) Further the urinary phosphorus nitrogen ratio was considerably higher in the groups receiving the tumour diets

This particular area of research appears to be promising If this phenomenon can be extended to other tumour host systems it would have a profound influence on present day concepts of the nutritional requirements of the tumour bearing host

3 Effect of Amino Acids

The amino acid lysine has long been recognized for its essential role in mammalian metabolism and this is no exception in tumour growth Voegtlin and his associates (Voegtlin and Thompson 1936 Voegtlin and Maver 1936) were the pioneer investigators in this field They prepared lysine deficient diets (by the inclusion of gliadin as the source of protein or heat treatment of whole milk powder) and noted that these diets inhibited the growth of spontaneous mammary carcinomas in mice Supplementing the diets with lysine or substitution of a complete protein for gliadin resulted in normal tumour growth Kocher (1944) also examined the effects of lysine on tumour growth He noted the same growth retarding effect as Voegtlin *et al* but this effect was not permanent If the lysine deficient diet was continued for a longer period the inhibited tumour growth gave way to a resumption of rapid growth equal to that of the control animals From this he concluded that lysine may be obtained by the tumour from body protein reserves or break down products of body tissue since there was a carcass weight loss of the animals during the latter stages of tumour growth

Voegtlin and co-workers (Voegtlin Johnson and Thompson 1936 Morris and Voegtlin 1940) also examined the effects of the sulphur-containing amino acids on tumour growth and reported that diets low in cyst(e)ine and methionine retarded the growth of spontaneous mammary carcinomas Following the period of tumour growth inhibition dietary supplements of cyst(e)ine glutathione or methionine exerted a striking stimulatory action upon tumour growth Allison and his associates (Allison *et al* 1955 1956) reported that supplements of dietary methionine in sarcoma bearing rats favoured carcass weight gains but methionine and cyst(e)ine were reported to reduce the growth of sarcoma R 1 (Hilf 1956) When excessive amounts of methionine were administered (Ghadially and Wiseman 1956) the rate of growth of the tumour was stimulated Dunn and his group (Levy *et al* 1953 Dunn and Murphy 1955 Murphy and Dunn 1957) have shown that ethionine (an antagonist for methionine) was inhibitory for several rat tumours

Antagonists for phenylalanine have been shown to affect tumour growth When β_3 thienylalanine was administered to rats bearing well-established Jensen sarcomas the growth of the tumour was inhibited with a reduction of mitosis nuclear changes and an

increase in necrosis (Wissler *et al* 1956) This effect could be overcome by increasing the amount of phenylalanine in the ration Koller and Veronesi (1956) administered a nitrogen mustard derivative of phenylalanine to rats bearing the Walker carcinosarcoma 256 and to mice inoculated with the Landschütz ascites tumour The L isomer was more effective than the D-isomer or the racemic mixture in inhibiting tumour growth, and mice bearing the ascites tumour survived a longer period of time than the controls

Azaserine a specific antagonist for glutamine has been shown to inhibit tumour growth as well as a number of other amino acid antagonists Most of these investigations however may be more conveniently classified under the subjects of pharmacology metabolism or chemotherapy This is also true of a number of other antagonists not mentioned in the present discussion

White and Belkin (1945) studied the effect of a low nitrogen diet on the growth of a transplanted adenocarcinoma (mammary) of the mouse The protein in the diet was limited to the small amounts contained in the 5 per cent liver extract supplement Interestingly enough the establishment of these tumours was not affected by these conditions but tumour growth was retarded Green Benditt and Humphreys (1950) conducted a similar series of investigations with rats bearing the Walker carcinosarcoma While the establishment of the implants was retarded as a result of protein depletion the growth of the established tumours was relatively unaffected A similar effect on the establishment of the Walker tumour was reported by Devik *et al* (1950) They observed that the inflammatory reaction during the period of the tumour 'take' persisted for a shorter period of time in rats on a 20 per cent protein diet than in those receiving 5 per cent dietary protein

Some mention should be made concerning the non essential amino acids In this laboratory rats bearing the Walker carcinosarcoma were given a diet containing 18 per cent lactalbumin as a source of protein Supplementing the diet with from 2 to 10 per cent of each of the non-essential glycogenic amino acids resulted in no effect on tumour latency or growth but carcass weight gains were improved Supplementing with the other non-essential amino acids had a similar effect (this laboratory unpublished)

4 Effect of Fat

Apparently the level of dietary fat has no effect on the growth of the tumour *per se* This has been demonstrated in transplanted tumours induced sarcomas (Baumann *et al* 1939 Tannenbaum 1942*b*) and spontaneous mammary carcinomas (Silverstone and Tannenbaum, 1950) Further the mean survival time of experimental mice (after the tumour has appeared) is unaffected by the amount of fat in the diet It has been mentioned that tumour bearing rats given a high fat diet *ad libitum* sometimes developed a transient lipaemia but this did not have any predictable relation to the growth of the tumour (Frederick and Begg 1946)

Haven Mayer and Bloor (1957) administered dietary tumour phospholipid to rats bearing the Walker tumour for a ten-day period when the rats had reached a plateau in body weight The tumour phospholipid in the diet seemed to induce anorexia and decrease carcass weight and appeared to favour tumour growth Normal animals on the same diet were not affected with respect to appetite or carcass weight changes These findings merit further investigation

5 Effect of Vitamins

Morris and Robertson (1943) studied the effects of dietary riboflavin on the growth rate of spontaneous mammary tumours in mice When the level of riboflavin was adequate for maintenance no appreciable effect was noted on tumour growth but a severe riboflavin deficiency decreased the rate of growth of the carcinomas Stoerk and Emerson (1949) also investigated riboflavin deficiency on established lymphosarcoma (6C3H ED) implants The deficiency was induced by either feeding a diet low in the vitamin or by the administration of an antagonist In all cases marked regression of the lymphosarcomas occurred as a result of riboflavin deficiency and in most cases survival of the mice was significantly prolonged Riboflavin deficient animals which had survived 60 days or more were re inoculated with the lymphosarcoma tissue but the implants failed to take

Antagonists of vitamin B_6 deoxypyridoxine hydrochloride and deoxypyridoxine phosphate, did not affect the growth of sarcoma 180 in mice unless the mice were maintained on a pyridoxine-deficient diet (Brockman *et al* 1956) A combination of deoxypyridoxine phosphate and acid hydrazide severely restricted sarcoma 180 growth when the mice were given a B_6-deficient diet Diets deficient in pantothenic acid and niacin have also been shown to retard the growth of spontaneous mammary carcinomas and adenocarcinoma 755 respectively in mice (Morris and Lippincott 1941 Shapiro *et al* 1957)

The folic acid requirement for the growth of the Rous sarcoma has been reported by Little Oleson and SubbaRow (1948) and some folic acid antagonists have been reported to inhibit some mouse leukaemias and other tissues Nevertheless these effects may be largely concerned with the establishment of the tumour and not with tumour growth as pointed out by Kirschbaum *et al* (1950) since aminopterin and

amethopterin had no effect on some transplantable leukaemias if they had been allowed to proliferate for about ten days The effect of dietary choline has usually been concerned with the genesis of tumours (previously discussed) and apparently it has no effect on tumour growth (Hill and Garren 1956)

Robertson *et al* (1949) observed that the growth of a transplanted fibrosarcoma was less in guinea pigs on a scorbutigenic diet The animals were placed on the diet about ten days after tumour implantation and the growth retarding effect on the tumour was demonstrable before changes in food consumption or body weight gains were noted The ascorbic acid content of the tumours was found to be essentially zero and the tumour collagen concentration was about one half that of the sarcomas grown in the controls This latter observation may prove to be a fertile field of investigation

IV CANCER IN MAN

Attempts to relate dietary influences to cancer in man are at best hindered by many inherent difficulties Most of this information is based on statistics but even here complications arise such as the time interval of observation number of cases observed and the incompleteness or inaccuracy of records This in addition to some unavoidable influences *i e* heredity sex degree of exposure to carcinogenic stimuli time required for the malignant transformation to occur etc makes it very difficult to come to any clear-cut decisions There are however a number of documents which imply certain relationships and these will be considered here

A Underfeeding Versus Overfeeding

Hindhede (1925) investigated the cancer deaths in Denmark and pointed out that the death rate was less when dietary rationing was in force (1917–19) than during the five year period after it was abolished From this he concluded that overfeeding was respon sible for the increase in the cancer death rate One of the principal objections to Hindhede s survey has to do with the length of time considered in the survey He pointed out that the Danish people were noted for their rich daily diet and it is difficult to comprehend how a severe rationing regime could inhibit the deve- lopment of tumours in the short period of one two or even three years At the same time it is difficult to see how the abolishment of rationing (in the 1920–25 period) could so rapidly reflect an increase in cancer deaths

Poscharissky (1930) and Peller (1934) reported a similar relation of dietary rationing to the incidence of gastric cancer in Russia At the end of World War I these restrictions were lifted and the number of gastric tumours increased While these two surveys covered a much longer period of time there may still be some questions raised concerning the relatively rapid increase in gastric cancer after the rationing period was terminated

In 1929 Dublin (1929) reported the results of insurance statistics relating overweight to cancer deaths He used as his source of data the Medico-Actuarial Mortality Investigation records of the Union Central Life Insurance Company and death claims of the Ordinary Department of the Metropolitan Life Insurance Company for 1927 These figures indicated that overweight men (50 pounds or more at the time the policy was issued and in the age group of 30–44 years old) showed a subsequent mortality from cancer of 37 per 100 000 normal weight men 32 per 100 000 and underweight men (25 pounds or more) had a mortality rate of 24 per 100 000 Similar relationships were demonstrated for age groups of 45 years or over

Another analysis of men purchasing insurance at the age of 45 years or over was provided from the records of the Union Central Life Insurance Company (192 000 records) When classified according to weight the cancer mortality per 100 000 was—25 per cent or more overweight 143 15–25 per cent overweight 138 5–15 per cent overweight 121 normal weight 111 5–15 per cent underweight 114 and 15–50 per cent underweight 95 From this it can be reasonably assumed that increasing weight can result in increasing mortality from cancer and in all probability in creasing cancer incidence These general findings have also been pointed out by Dublin and Marks (1937, 1938) Hoffman (1937) Hunter (1939) Tannenbaum (1940*b* 1947) and Armstrong *et al* (1951)

As may be expected the relation of body weight to cancer incidence may be correlated more closely with some tumour types than others and it appears that the incidence of neoplasms of the liver gall bladder intestines and urinogenital organs can be related to body weight to a greater extent than those in other sites (Dublin and Marks 1937 1938 Armstrong *et al* 1951)

Nevertheless it should be remembered that while cancer is more prevalent in overweight persons severe dietary restrictions will not necessarily prevent the formation of tumours nor will dietary rationing of a person afflicted with cancer inhibit the growth of an established neoplasm.

B Malnutrition

The incidence of primary liver cancer and its rela tion to malnutrition has probably received more attention from investigators than any other tumour

site This may be due to the fact that the frequency varies from 1 to 41 per cent in different regions of the world The natives of south-east Asia *i e* China Java Japan and Sumatra and particularly those of Uganda and South Africa (Bantus) have an exceptionally high frequency of liver carcinoma (7–41 per cent) (Bonne 1937 Berman 1940 1951, Gilbert and Gillman 1944 Gillman Gillman and Gilbert 1951 Oettle 1956, Oettle and Higginson 1956 Shanmugaratnam 1956) This disease however is surprisingly low in Caucasians (Pack and LeFevre 1930 Berman 1940) as well as in negroes living in the United States and Europe (Kennaway, 1944) From this it appears not to be a problem of racial susceptibility or endemic infection but more probably a question of malnutrition

Gillman and co workers (Gillman 1944 Gillman *et al* 1945) fed rats for an extended period of time on a mealy meal porridge and sour milk (the principal food of the Bantus) and reported severe liver damage and some cirrhosis in the experimental animals No hepatomas however were found In view of the fact that liver cirrhosis was frequently common in populations with a high incidence of carcinoma of the liver (Berman 1941 Gilbert and Gillman 1944) the hypothesis of the relation of malnutrition to frequency of hepatomas received much support

Cook Duffy and Schoental (1950) intermittently fed rats with alkaloids of *Senecio Jacoboea* and observed extensive liver damage leading to tumours of the liver in animals which survived at least ten months of treatment In addition rats fed chilli peppers also developed hepatomas (Hoch Ligeti 1951) Schoental (1953) extended the work of Cook *et al* (1950) and used *Senecio* alkaloids common in South Africa in additional feeding experiments These results confirmed the findings of Cook *et al* (1950) He pointed out that while these components are not common in the diet they are used widely in the native medicine of the Bantus for a variety of disorders throughout their life As a result malnutrition may render the liver vulnerable to a variety of pathological conditions —one being the induction of hepatomas in individuals consuming crude carcinogenic agents The possible harmful effects of the caffeine-containing cool drinks on children in South Africa has also been reported (Steyn 1956)

The incidence of carcinoma of the oral cavity pharynx, and oesophagus in women residing in the Arctic regions of Sweden and Finland is reported to be high These individuals usually have a history of Plummer–Vinson syndrome and it is believed to be related to a possible iron or vitamin-deficient diet Under Arctic environmental conditions reindeer meat and fish provide the principal diet and only during the short summer seasons are green vegetables available (Ahlbom 1936 Adair 1947)

In areas in which goitre is endemic malignancy of the thyroid seemed to be prevalent For example the incidence of tumours of the thyroid was much greater in Bern a goitrous area than in Vienna Prague or Berlin (Weglin 1928) Further Weglin in some histological studies observed that malignancy of the thyroid almost invariably proceeded from benign tissue proliferation This latter point was also suggested by Ward (1944) and Cole Slaughter and Rossiter (1945) Since endemic goitrous regions are characterized by lack of iodine in the soil drinking water and foods produced from the soil it would appear that chronic iodine deficiency may be a factor in the frequency of thyroid tumours

Tromp (1954) compared the cancer mortality rate in the Netherlands with a study of soil types of that area He noted high death rates in inhabitants living on a reclaimed peat soil and correlated various soil types with the cancer death rate This was extended to include the relation of gastric cancer to the soil (Tromp and Diehl 1955 Tromp 1956) but at the present status of our knowledge this information may be too difficult to interpret in terms of the cancer problem. It should be mentioned that Fournier (1956) did not indicate that the dietary regime in Morocco had any relationship to tumours of the digestive tract

In all as Cowdrey (1955) has pointed out, the relation of cancer incidence to dietary restriction in man cannot be expected to be elucidated until larger and more homogenous groups are investigated The records of the Veterans Administrations in various countries could be a fruitful source of information in the future

V SUMMARY

We have considered this problem from three stand points and certain conclusions can be drawn For simplicity s sake these are listed under their respective headings—Nutrition of Neoplasms *in Vitro* Nutrition of Neoplasms *in Vivo* and Cancer in Man

A Nutrition of Neoplasms *in Vitro*

The formulation of some chemically defined media for continuous cell growth in tissue culture has been accomplished These media however have only been demonstrated to support the growth of the Strain L, and only those developed by Earle s group support rapid growth In these latter studies the cells underwent some drastic changes during the conditioning period and it may be reasonably assumed that the nutrition and metabolism of that particular substrain was affected Nevertheless some notable advances have been made in this area

The refinement of clonal growth techniques has amply demonstrated that different substrains of cells can be readily isolated from a single neoplasm and that these clones differ in their nutritional requirements While the essential components for growth *in vitro* may follow a general pattern for different tumours and normal tissues many nutritional differences have already come to light and perhaps many more remain to be revealed Further it has been proved that the nutritional requirements of massive inocula may differ from those of dilute cell culture

B Nutrition of Neoplasms *in Vivo*

When one considers tumour development and growth *in vivo* the genesis of the neoplasm as contrasted with tumour growth seems to be more markedly influenced by dietary alterations Of these caloric restriction has the most striking effect on practically all experimental tumours Fat-enriched diets favour the formation of spontaneous mammary carcinomas some induced skin tumours and perhaps some hepatomas while low dietary protein (to the extent that an amino acid deficiency may be involved) may inhibit spontaneous hepatomas but enhance the development of the induced hepatomas The vitamin riboflavin appears to play a special role in the formation of hepatic tumours induced by *p* dimethylaminoazobenzene dyes

Caloric restriction and severe protein (or amino acid) deficiency may retard the growth of an established tumour but this inhibition is displayed at the expense of the host Variation in fat or vitamin content in the diet does not particularly affect tumour growth In all once the neoplasm is established growth appears to be more or less independent of dietary influences Of particular significance are the recent findings of Mider s group which suggest that the nutritional requirements for the well being of the tumour bearer may differ from that of the normal animal

C Cancer in Man

Obesity in man can be correlated with a higher incidence of cancer while there is evidence that malnutrition may increase the incidence of certain specific tumour types Nevertheless a more reasonable explanation is that malnutrition may render a specific organ or tissue vulnerable to some other carcinogenic stimuli For the most part it does appear that the nutritional status of the host is a modifier of cancer development and not the initiator of the carcinogenic process

D Concluding Remarks

Up to the present time the policy has been to consider nutritional aspects at the cellular level and at the host level two completely independent fields This is probably due to the large gaps in our present day knowledge despite the remarkable advances during the last three decades It only points out the critical need for more facts concerning this problem and a more precise understanding of the underlying reaction mechanisms involved When this is accomplished information may well be correlated and consummated into the complete and unified concept of the intimate relationships of nutrition to neoplasia

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The Use of Isotopes in Nutrition Research with Special Reference to Tritium

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I INTRODUCTION

Isotopes are now available for labelling a wide range of dietary constituents and the role of labelled nutrients in many vital processes of nutritional interest has been investigated However isotopic methods have been applied only infrequently to the investigation of such questions as the assessment of dietary requirements the availability of nutrients and the effects on the nutrition of organisms of different levels of dietary constituents It seems evident from results obtained in other fields where isotopic techniques are now well established that important contributions to the study of nutrition could be made should nutrition workers become more interested in the use of isotopes and workers with isotopes more interested in nutrition

A great deal of information obtained from isotopic studies of the metabolism of animals has of course a direct bearing on problems of nutrition but it would be profitless to discuss the contribution made by the use of isotope methods in isolation from results obtained by the use of other techniques We have however attempted to illustrate the way in which different isotopic methods of investigation have been applied to investigations of a wide range of problems related to nutrition

Brief reference is made first to the isotopes which may be applied to nutritional investigations investigation of the total amounts of certain body constituents and the nature of their distribution are discussed in the following section The main subject of later sections is the use of isotopes to obtain direct evidence of the fate of nutrients investigations of the rates of change of metabolic processes are referred to only occasionally since the basic criteria for this type of work are still under discussion (for example Reiner 1953*a* *b*)

Finally we discuss the use of tritium as a label and methods which have been developed in this laboratory for its application to nutritional investigations

II ISOTOPES USED IN NUTRITION RESEARCH

A list of some of the isotopes which have been used as tracers in nutritional research is given in Table 9 1 together with estimates of the accuracy and sensitivity achieved with assay procedures in general use Accuracy and sensitivity are given in terms of that quantity of isotope which gives a reading equal to the background reading Figures are also given in the table for the maximum dilution of the highest specific activity material available (*A E R E Catalogue of Radioactive Materials* 1957)

Thus the table gives some idea of the relative utility of different isotopes as applied to problems involving accurate dilution measurements When the behaviour of a particular chemical element is being studied there may be little or no choice of isotopes and a decision has only to be made between alternative assay methods Considerations which govern such a choice are discussed in a later section (VI) When a molecule is to be labelled or tagged there may be a wider choice of isotopes For example proteins have been labelled with isotopes of carbon hydrogen nitrogen sulphur and iodine Considerations which lead to the selection of a particular isotope for such a problem are firstly availability at high specific activity at reasonable cost and secondly the existence of a sensitive and accurate routine method for assay Additional considerations are that the half life of the isotope should be long in relation to the duration of the experiments contemplated but not so long as to seriously limit the maximum available specific activity the radiation emitted should not be such as to cause serious health hazards in handling or impose a low limit to the amount of labelled material which could be administered to an experimental animal without fear of a disturbance of normal metabolism The isotopes of hydrogen and carbon are of the most general use for labelling organic molecules The stable isotopes ^{2}H and ^{13}C may be used but the dilutions measurable are very low (see Table 9 1) compared with those attainable using the radioactive isotopes ^{3}H and ^{14}C

^{14}Carbon has a half life of 5 600 years High specific activity material is expensive but a large range of labelled compounds is available Methods for assay in general use are given in the table Bearing in mind the special difficulties associated with all these the very high sensitivity and accuracy of the gas counting method point to this as the best for routine use

Tritium has a half life of 12 years and very high specific activity material is available at low cost It has been traditionally regarded as a difficult isotope to use for tracer work probably because solid counting with an end window Geiger counter is not possible However several ion chamber liquid scintillation and gas counting procedures are now in use and solid counting at low efficiency is possible using a window-less gas flow counter In conjunction with a gas counting method higher dilutions of tritium can be measured than of any other isotopes

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TABLE 9 1

Isotopes used in the Study of Nutritional Problems together with Methods of Measurement in General Use

(Approximate figures are given for reproducibility and sensitivity in terms of background equivalent activity)

Isotope	Assay method	Sensitivity (B E A.)	Accuracy (per cent)	Usable dilution ratio
^{2}H	Density determination	—	—	10^{4}
	Mass spectrometric determination	—	±0 5	10^{4}
^{3}H	Solid counting using *windowless flow counter*	—	±15	—
	Liquid scintillation counting	0 001 μc	—	10^{3}
	Gas phase assay 1 Ionization chamber *hydrogen filling*	0 01 μc	±5 for amounts > 0 05 μc	10^{4}
	2 Geiger counter butane filling	—	—	—
	3 Proportional counter methane filling	0 0000? μc	±1	10^{5}
C	Mass spectrometric determination	—	±0 5	10^{4}
^{1} C	Solid counting using *thin* end window counter or preferably windowless proportional gas flow counter	0 0001 μc	±4	10
^{14}C	*Liquid scintillation coun*ter carbon incorporated into scintillator, or dissolved as benzoic acid	*0 0005 μc*	*Good at* high levels	*10*
	Gas phase assay as CO_2 *in proportional counter*	0 00001 μc	±1	10^{8}
^{15}N	Mass spectrometric determination	—	±0 5	10^{4}
O	Mass spectrometric determination	—	±0 5	10^{4}
^{2}Na	Well type scintillation counter	0 00001 μc	—	10
	Liqu d Geiger counter	0 0001 μc	±1	10^{4}
Na	Well type scintillation counter	0 00001 μc	—	10
	Liquid Geiger counter	0 0001 μc	±1	10
P	Sol d sample counting with thin end window *counter or* windowless gas flow proportional counter	0 0001 μc	—	10^{11}
	Liquid sample using well type Geiger counter	0 0001 μc	*±1*	*10^{3}*
S	Sol d sample count ng windowless gas flow proportional counter	0 0001 μc	±4	10
^{3} *Cl*	Solid sample counting end window	0 0001 μc	—	10
^{42}K	End window counter or *well type scintillation* counter	0 0001 μc	—	10
	Liquid Geiger counter	*0 000 μc*	±1	10
Ca	Sol d sample counting with thin end window or windowless gas-flow proportional counter	0 001 μc	±4	10
		0 000 μc	±4	10
Fe	X ray counter well type scintillation counter or liquid scintillation counter	—	—	—
Fe	End window counter or well type scintillation counter	0 001 μc	—	—
Co	End window counter or well-type scintillation counte	0 0005 μc	—	10^{4}
	Liquid Ge ger counter	*0 001 μc*	±1	10
^{6}Cu	End window counter	0 0002 μc	—	*10*
	Scintillation well counter	0 005 μc	—	*10*
Zn	X *ray* counter or well *type scintillation counter*	—	—	—
^{89}Sr	End window counter or *well type scintillation* counter	—	—	—
^{13} I	End window counter or well type scintillation count r	—	—	—
^{1}I	End window counter or well type scintillat on counter	0 000 μc	—	10
	Liquid Geiger count r	0 001 μc	±1	10^{11}

III INVESTIGATIONS EMPLOYING THE PRINCIPLE OF ISOTOPE DILUTION

This type of investigation is applicable to constituents known to be (uniformly) distributed in vascular or vascular plus extravascular spaces The principle of this method is by no means new it is the same as that employed in the widely used dye method for the determination of plasma volume where the dilution observed following the injection of a known amount of dye allows the volume of *circulating* plasma to be calculated Errors result if the isotope is not homogeneously *distributed* or if loss or transformation occurs before complete mixing has taken place and a pure sample obtained

1 WATER

Hevesy and Hofer (1934) first applied deuterium as a tracer in an investigation of the rate of movement and the total amount of water in a human subject Pace *et al* described the preparation and use of tritium labelled water in 1947 and nowadays both hydrogen isotopes are frequently used in body water studies Recently Anbar and Lewitus (1958) used water labelled with three isotopes deuterium tritium and ^{18}O in studies of body water in rabbits No difference was found in the rate of increase of concentration of the isotopes in the blood and the dilution at equilibrium was the same in each case However the rate of loss of the oxygen isotope was the most rapid (half life 80 hr) The authors suggest that this may be due to an additional route of excretion by oxidation Marked differences were found in the rates of loss of the hydrogen isotopes (half life for deuterium 220 hr for tritium 130 hr) It was suggested that this isotope effect may have been operative at a stage of excretion involving proton transfer

2 CHLORIDE

^{38}Cl half life about 37 min was prepared (Winkler Elkinton and Eisenman 1943) by the bombardment of lithium chloride with deuterons in a cyclotron and had to be used immediately after preparation When the long life isotope ^{36}Cl became available much more extensive work was possible Burch Threefoot and Ray (1950) were able to carry out experiments of duration up to one month on dogs which had received a single injection of labelled chloride They found that the amount of chloride passing out of and into the serum per day expressed as sodium chloride was on the average 1 2 times the weight of the animal Mean chloride space was 35 per cent of the body weight and half life about 3 5 days These studies were subsequently extended to humans (Threefoot Burch and Ray 1953) Theoretical considerations concerning this type of work were discussed by Burch Threefoot and Cronwich (1949)

Gable *et al* (1953) found that ^{38}Cl and ^{82}Br had a similar distribution in man Since ^{82}Br administration does not involve the same biological hazard as ^{36}Cl it has been used as a marker for chloride Forbes *et al* (1953) were thus able to show that total exchangeable chloride for newly born children was 51 1 meq/kg compared with 32 meq/kg and 26 meq/kg for young men and young women respectively found by Reid *et al* (1956)

3 SODIUM

The equilibration of ^{24}Na in rabbits was studied by Edelman and Sweet (1956) Total exchangeable sodium was 46 0 meq/kg 14 per cent of which was recovered from the gastro intestinal tract chiefly in the proximal half of the small intestine Similar results were obtained when the animals were fasting Forbes and Perley (1951) measured total exchangeable sodium in humans and found values ranging from 32 to 54 meq/kg Determination of sodium specific activity in bone suggested that as much as 60 per cent of skeletal sodium was metabolically inert in the sense that it was not free to equilibrate with sodium in tissue fluids Veal *et al* (1955) described a method of determination of total exchangeable sodium in man employing ^{22}Na and a whole body counter

4 POTASSIUM

The short lived isotope ^{42}K was used by Corsa *et al* (1950) for the measurement of exchangeable potassium in man Exchange rates for different tissues varied in experiments on both animals and humans—equilibration was rapid in viscera fairly rapid in muscle slow in brain and erythrocytes and probably slow in bone Samples of urine potassium were representative at 40 hr the time of equilibration The mean total exchangeable potassium for healthy young men was 46 3 meq/kg It was estimated that total exchangeable potassium was about 5 per cent less than total body potassium but Rundo and Sagild (1955) considered that there was a greater difference The latter workers measured total potassium by means of a total body γ-monitor it was assumed that the radiation recorded was due to the natural isotope ^{40}K Woodward *et al* (1956) also used total body γ-radiation as a measure of total potassium Good correlation of activity and fat free body weight was shown Nadell Sweet and Edelman (1956) used a $^{42/43}$K mixture produced by α particle bombardment of argon and found that in rabbits equilibration was virtually complete after 40 hr but not complete after 24 hr The rate of faecal excretion of potassium was five times the rate of sodium excretion and it was estimated that 80 per cent of the total extracellular potassium (7 2 per cent of the total potassium) was in the gastro intestinal tract

IV INVESTIGATIONS OF THE FATE OF SOME MINERAL NUTRIENTS

An isotope of the element concerned is used as a label in this type of investigation which is thereby distinguished from investigations of organic nutrients

1 IRON

Work with isotopes of iron has helped to elucidate some aspects of the relation of absorption transport [illegible] storage of iron to the requirements of organisms

Hahn *et al* (1939*a*) used radioactive iron to study absorption by anaemic dogs It was shown that whereas normal dogs absorbed only negligible amounts the isotope was quite promptly absorbed by anaemic dogs Excretion was studied later (Hahn *et al* 1939*b*) Observation of the very low iron excretion in faeces by dogs was in agreement with the results of McCance and Widdowson (1938) who had carried out balance experiments on human subjects given different levels of oral and intravenous iron

Hahn *et al* (1943) found that 5–15 times the normal low absorption of iron took place after dogs had been made anaemic by bleeding Active absorption was found in pouches of stomach duodenum and jejunum The importance of the mucosal epithelium of the gastro-intestinal tract in regulation of iron absorption was confirmed Absorption of labelled Fe was greatly reduced when inactive iron had been fed 1–6 hr earlier which seemed to indicate that absorption was limited by the capacity of the epithelium to admit iron—that is a mucosal block was operative Chodos *et al* (1957) found that the mucosal block did not always prevent excess iron accumulation These workers developed techniques for the preparation of ^{59}Fe labelled eggs and vegetables and investigated absorption from those foods and from iron salts by normal and iron-deficient subjects and also absorption by patients with idiopathic haemochromatosis Absorption from ferrous chloride occurred more readily and in greater amount in all cases and this difference appeared to be largely due to dietary factors which could modify the form and solubility of iron in the lumen of the gastro intestinal tract However it was found that food iron was absorbed and used to a much greater extent by patients with iron deficiency than by normal subjects In addition to such evidence of a complex mechanism whereby iron absorption is regulated in relation to the requirements of the organism it appears that the re utilization and use of stored iron is also complicated Iron from all breakdown or from recently absorbed or injected labelled salts does not equilibrate with stored iron, but appears to be more readily available for metabolic needs (Bothwell *et al* 1956)

Determination of plasma iron and erythrocyte iron turnover became possible with the availability of high specific activity ^{59}Fe Huff *et al* (1950) found that plasma iron turnover in normal man was about 1½ times that required for renewal of red-cell iron at a rate of 0 85 per cent per day

Jensen *et al* (1956) studied the kinetics of iron metabolism in normal growing swine The value determined for the average red cell life span was 63 days in agreement with that found by Bush *et al* (1955) using ^{14}C Analysis of the ^{14}C results showed that, in this species erythrocytes were destroyed by both a random and an age-dependent process Berlin *et al* (1957) found good agreement between the results of three methods of determination of red cell life span in humans cell survival was determined using ^{59}Fe and [^{14}C]glycine and the Ashby differential agglutination technique

Two iron isotopes were used in a method devised by Saylor and Finch (1953) for investigation of the absorption of iron by rats One isotope ^{55}Fe was added to the food the second isotope ^{59}Fe was incubated with plasma and then injected intravenously Later, the amount of each isotope in the blood was determined The change of activity of ^{59}Fe gives a measure of the rate of removal of iron from the blood The amount of iron absorbed can then be calculated from the ^{55}Fe activity

The remarkable extent of the persistence in the human infant of ^{55}Fe derived from the mother was demonstrated by Smith *et al* (1955) Results indicate that little or no utilization of dietary iron for haemoglobin formation occurred until 3 to 4 months after birth At the age of 2 years iron from the mother still constituted about 40 per cent of the total haemoglobin iron and over 90 per cent of the iron present at birth still remained in haemoglobin

2 Copper

Balance studies of the metabolism of copper are difficult, but recently information on the absorption and metabolism of dietary copper has been obtained by the use of ^{64}Cu Thus it has been shown that only 30–40 per cent of small (1 mg) doses of copper are absorbed (Gubler 1956) Mahoney *et al* (1955) concluded that dogs have only a limited capacity for the excretion of copper the greatest amount being excreted in bile The rate of arrival of administered ^{64}Cu at different sites has been recorded by placing counter tubes at appropriate positions on the body surface It has been found in this way that the liver takes up most of the administered dose Injected ^{64}Cu appears first in the albumin fraction then after 4–6 hr in the globulin fraction of the serum proteins as ceruloplasmin (Gubler 1956) Erythrocytes also have a labile and a stable ^{64}Cu fraction (Bush *et al* 1956)

3 Cobalt

The nutritional importance of cobalt became clear with the recognition of widespread cobalt deficiency among ruminants Investigation of the absorption, excretion and distribution of this element were difficult, because of the extremely small amounts involved, but these difficulties were overcome to a great extent by the use of radioactive cobalt isotopes Comar and Davis (1947) used a preparation of cobalt isotopes prepared by bombardment of iron with deuterons. 80 per cent of the cobalt administered orally to cattle

appeared in the faeces 65 per cent was excreted in the urine following intravenous administration and 30 per cent in the faeces Braude *et al* (1949) investigated distribution of ^{60}Co in tissues of pigs following a six week feeding period on a diet containing physiological amounts of labelled cobalt Evidence of storage in the liver was obtained and the results for distribution in the tissues were in most cases similar to those obtained by Comar and Davis (1947) from a pig following a single oral dose

Vitamin B_{12} labelled with ^{60}Co has been prepared and its fate determined following administration of microgram quantities Halsted *et al* (1956) report the results of a comparison of different absorption tests employing ^{60}Co Special methods were developed for routine assay of faecal samples in elimination tests However the test devised by Schilling (1953) was considered preferable In this a measure of the absorption of an orally administered dose is obtained by subcutaneous administration of 1 mg of non radioactive vitamin B_{12} and measurement of the radioactivity of the urine following this flushing dose Previously, the only method of detection of B_{12} absorption had been the haematological observation of response by deficient subjects The introduction of labelled vitamin B_{12} made possible absorption studies on other subjects Vitamin B_{12} labelled with ^{56}Co ^{57}Co and ^{58}Co isotopes has been prepared (Mollin and Lester Smith 1956) in order to reduce the radiation exposure resulting from absorption tests using the ^{60}Co isotope Okuda Grasbeck and Chou (1958) studied the excretion of B_{12} by rats A considerable fraction was excreted in the bile in a bound form Further excretion took place from the intestinal wall so that radioactivity observed in samples of faeces following oral administration of labelled vitamin B_{12} is not entirely due to unabsorbed vitamin

4 *Calcium*

^{45}Calcium was first produced by Walke (1940) and shortly afterwards Campbell and Greenberg (1940) used it in a quantitative study of the fate of calcium salt administered to a rat 80 per cent of the dose was absorbed 67 per cent being subsequently excreted in the urine The distribution of the retained isotope in the tissues was determined and highest specific activities were found in teeth and bone The kinetics of ^{45}Ca in body fluids is at some stages dominated by the influence of the relatively large mass of bone calcium The crystals of bone mineral being extremely minute their surface area is very high Robinson (1952) showed that the specific surface was of the order of 100 m^2/g so that a high proportion of crystal calcium ions are at or close to the surface Comar (1956) discusses the ion-exchange effect in relation to ^{45}Ca *investigations* Harrison and Harrison (1950) considered that incorporation of calcium into the skeleton was not a measure of bone salt formation because of the dominating influence of ion-exchange Essentially all the bone calcium of young rats was exchangeable with body fluid calcium and the proportion of exchangeable skeletal calcium was much lower in older rats The rate of turnover of skeletal calcium was apparently increased by administration of vitamin D but not by changes in dietary calcium intake

A method has been developed for measurement of the absorption of calcium by livestock under different physiological and dietary conditions in which allowance may be made for the large endogenous calcium excretion and the heterogeneity of bone calcium (Visek *et al* 1953) Comar (1956) reported that both calves and older animals were able to absorb and retain more calcium from milk than from hay or concentrate Blau *et al* (1954) found 4 per cent and 67 per cent utilization of labelled calcium by human subjects

5 *Phosphorus*

The first experiment in which a radioactive indicator was employed was described by Chiewitz and Hevesy in 1935 Rats were given traces of ^{32}P labelled phosphate and the absorption excretion and distribution of the phosphate were studied Thus there is a relatively long history of the application of this isotope Various tracer techniques of general application were initially developed in the course of work with ^{32}P recognition of the importance of organic phosphate compounds in intermediary metabolism led to their use in studies of a diversity of problems

The effect of reduced phosphate intake on the assimilation of $^{32}PO_4$ was investigated by Gaunt, Griffith and Irving (1942) A high retention of the $^{32}PO_4$ by the skeleton was observed in a group of rats fed a diet containing 0 3 per cent of Ca and P but a group fed a diet containing 0 12 per cent of Ca and P retained more $^{32}PO_4''$ per unit weight of skeleton Kleiber *et al* (1951) determined the endogenous phosphate excreted by cows It was found that when a steady state of phosphate movements had been established by a series of $^{32}PO_4$ injections the ratio of endogenous phosphate to total phosphate in the faeces was equal to the ratio of the mean specific activity of the faeces to the mean specific activity of the plasma two days earlier Endogenous phosphate being known 'true digestibility could be calculated In one experiment apparent digestibility was 12 per cent true digestibility 50 per cent, in another, apparent digestibility was negative true digestibility 64 per cent Hevesy (1948) in his survey of applications of radioactive indicators describes measurement of the endogenous phosphate of humans The specific activity of the urine phosphorus was taken as an index

of the specific activity of phosphate secreted into the intestinal tract

Weidmann (1956) studied the rate of renewal of phosphate in the long bones of experimental animals The different rates found for different species and the time relations of phosphate uptake did not appear to be explicable on the basis of ion exchange at the surfaces of crystals

A recent example of nutritional interest of the very numerous investigations of the kinetics of organic phosphate compounds is the work of Shore Zilversmit and Ackerman (1955) on phospholipid synthesis in experimental atherosclerosis in rabbits It was found that at least 90 per cent of the phospholipid appearing in the aorta was synthesized *in situ* and not taken from the plasma

Dawson (1957) points out that, whereas the formulations of Zilversmit Entenman and Fisher (1943) for the calculation of phospholipid turnover in animal tissues have very often been applied the experimental conditions have hitherto never satisfied the theoretical criteria The chief reasons given for this were that the immediate precursors of the compounds under investigation were not known and that available analytical techniques were not adequate It is therefore difficult to assess the degree of approximation of estimations of the turnover of individual phospholipids in tissues However rapid turnover is clearly indicated and Dawson (1957) discusses their dynamic biochemistry which was initiated by the work of Hahn and Hevesy (1937) on lecithin synthesis in brain tissue of adult animals

Hevesy (1948) has reviewed studies of nucleic acid turnover in rats especially high rates were found in tissues of young rats and in the intestinal mucosa of mature animals

6 Iodine

Schiff *et al* (1947) using labelled iodide showed that the concentration of iodide in human saliva and gastric juice was 20–30 times that in the plasma Absorption of $Na^{131}I$ from the gastro-intestinal tract of rats was investigated by Albert Tenney and Lorenz (1952) Little NaI was absorbed from the stomach but a major portion of NaI placed in the small or large intestine was absorbed and re secreted by the stomach Thus apparently there exists an enteric circulation of iodide The amount of iodide concentrated in gastric juice and saliva would account for a considerable portion of the iodide space

Since chemical methods were unsatisfactory for estimation of the very low iodine content of plasma urine and faeces in rats fed a low iodide diet Van Middlesworth (1956) developed a method for iodide balance studies using ^{131}iodide labelled diet It was shown that on a low iodide diet iodide was excreted mainly in the faeces That faecal iodide represented excreted rather than unabsorbed iodide was indicated by the results of determinations of the faecal activity following parenteral administration of labelled iodide

It has been possible to study the nature of plasma iodine which usually amounts to only about 5 μg/100 ml Thus Taurog and Chaikoff (1948) obtained evidence that circulating thyroid hormone in the normal animal consists of thyroxine loosely attached to plasma protein and Gordon *et al* (1952) demonstrated the association of circulating labelled thyroxine with a particular plasma globulin fraction Gross and Pitt Rivers (1952) identified 3 5 3 L triiodothyronine in human plasma

The theoretical and practical considerations involved in the use of ^{131}I labelled plasma proteins have been discussed in a recent review by McFarlane (1957*a* 1957*b*)

7 Sulphur

Rapid excretion of labelled sulphate by man was reported by Borsook in 1937 Dziewiatkowski (1949) studied sulphate excretion and distribution using rats 95 per cent of the dose was excreted in urine and faeces in 120 hr Activity increased up to the 8th hour in bone and up to the 24th hour in bone marrow in contrast to the rapid fall in activity in blood and liver Recently the localization and the nature of retained sulphate has been examined in detail especially by autoradiography Bostrom and Jorpes (1954) summarize results of a systematic study of sulphate fixation in normal tissues of rats and rabbits High activity was found in cartilages the epithelial layer of the gastro-intestinal tract aorta heart valves cornea sclera of the eye and in mast cells Labelled chondroitin sulphate was isolated

A considerable amount of the sulphate incorporated into cartilage was considered by Davies and Young (1954) to exist in some form other than free chondroitin sulphate These workers showed activity to be highest in the zone of cell columns of epiphyseal cartilage especially in young rats

Glucksman Howard and Pelc (1956) found high incorporation of $^{35}SO_4$ in the fibroblasts and growing fibres of wounds in contrast with the low incorporation into the fibrous clot and scab Kodicek and Loewi (1955) found that decreased incorporation of sulphate into granulation tissue occurred in scorbutic animals

^{35}S is a particularly suitable label for compounds which are characterized by the presence of sulphur e g methionine cystine taurine and thiamin. Labelled proteins of specific activity sufficiently high for further study have been prepared from animals to which ^{35}S labelled yeast protein has been administered (Schultze and Maurer 1957)

V INVESTIGATIONS OF THE METABOLISM OF MAJOR ANIMAL NUTRIENTS USING ISOTOPES OF HYDROGEN, CARBON AND NITROGEN

1 CARBOHYDRATES

(a) *Absorption*

Atkinson Parsons and Smyth (1957) studied the absorption of [14C]glucose from intestinal loops of dogs They point out the difficulties involved in investigations of glucose absorption by chemical analysis it is necessary to introduce glucose solutions of high concentration into the lumen if differences between arterial and portal blood are to be measurable and furthermore it is by no means certain that all the glucose in portal blood has come from the lumen Atkinson *et al* (1957) found that 70–80 per cent of the absorbed glucose appeared as such in mesenteric venous blood 7–17 per cent as lactic acid and only insignificant amounts as carbon dioxide alanine and pyruvic acid Glucose was absorbed against a concentration gradient also absorption involved only a unidirectional movement of the glucose since the specific activity of glucose in the lumen did not decrease during absorption

The immediate fate of absorbed fructose in rats and guinea pigs was compared by Kiyasu and Chaikoff (1957) Guinea pigs converted about 70 per cent of the fructose to glucose in rats as much as 60 per cent of the activity in portal plasma was in the form of lactate most of which was formed in the intestine prior to absorption Only about 10 per cent of the absorbed activity was recovered as glucose from rats and up to 30 per cent occurred in unidentified compounds

(b) *Glucose Oxidation*

Wyshak and Chaikoff (1953) have studied the impaired carbohydrate utilization by liver slices from animals fasted 48 and 72 hr Oxidation of glucose to CO_2 was depressed by as much as 50 per cent but the amount of $^{14}CO_2$ obtained from fructose was not appreciably altered indicating that the defect in glucose utilization occurred at the stage of the initial phosphorylation reaction Lipogenesis from both glucose and fructose was impaired by fasting

Glucose oxidation involving reactions other than those of the Embden–Meyerhof pathway are known to occur in some micro organisms Wood (1955) reviewed their significance and discussed the possibilities of differentiating different pathways of glucose utilization in higher animals Murphy and Muntz (1957) found that 56 per cent of labelled glucose in blood perfused through rat liver was oxidized via the phosphogluconate pathway but they point out that only oxidative reactions were followed in their experiments and that other synthetic reactions could have taken place Marks and Feigelsen (1957) found that pentosephosphate reactions were more important in livers of fasting animals The relatively minor role of glucose in the energy metabolism of lactating cows was demonstrated by Baxter Kleiber and Black (1955) Only about 10 per cent of the CO_2 exhaled was obtained from glucose

(c) *Lactose Synthesis*

Pazur and Tipton (1957) found that intact galactose and glucose were used for lactose formation by guinea pigs The mechanism of lactose synthesis *in vitro* by cows and a goat was investigated by Schambye Wood and Kleiber (1957) The glucose moiety appeared to be derived from free glucose and the galactose from hexose phosphate esters The complex distribution of activity in galactose following administration of 14C labelled bicarbonate and acetate was considered to result from the synthesis of this moiety in the mammary gland Duncombe (1957) demonstrated net synthesis of lactose from [14C]glucose by mammary gland slices of guinea pigs rats and sheep using a paper chromatography technique The glucose and galactose from rat lactose were both radioactive No active lactose was detected following incubation with [14C]pyruvate

(d) *Glycogen Synthesis*

Labelled glucose appeared to be incorporated intact into muscle glycogen in both fed and fasted rats (Marks and Hyelem 1957) Stetten and Stetten (1954) from a study of the nature of glycogen regeneration in rats established the metabolic heterogeneity of the glycogen molecule The distribution of [14C]glucose in liver and carcass glycogen was investigated by exhaustive β amylase degradation in this way maltose was removed from the unbranched peripheral tier of glucose units leaving a dextrin core Activity was found mainly in the peripheral region at first but an increasing fraction was found later in the dextrin core by the 12th hour dextrin activity was higher than peripheral activity in glycogen from liver but in samples of glycogen from carcass most of the activity was in the peripheral region Fasting had little influence on the incorporation of glucose into carcass glycogen in contrast to the marked effect on liver glycogen where activity became nearly evenly distributed 3 hours after injection of [14C]glucose The doubtful meaning of half lives of glycogen molecules and the difficulty of defining their turnover are discussed

2 FATS

The first exploratory biological experiments with tracers described by Schoenheimer and Rittenberg

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Since chemical methods were unsatisfactory for estimation of the very low iodine content of plasma, urine and faeces in rats fed a low iodide diet, Van Middlesworth (1956) developed a method for iodide balance studies using ^{131}iodide labelled diet It was shown that on a low iodide diet, iodide was excreted mainly in the faeces That faecal iodide represented excreted rather than unabsorbed iodide was indicated by the results of determinations of the faecal activity following parenteral administration of labelled iodide

It has been possible to study the nature of plasma iodine which usually amounts to only about 5 μ 100 ml Thus Taurog and Chaikoff (1948) obtained evidence that circulating thyroid hormone in the normal animal consists of thyroxine loosely attached to plasma protein, and Gordon *et al* (1952) demonstrated the association of circulating labelled thyroxine with a particular plasma globulin fraction. Gross and Pitt Rivers (1952) identified 3 5 3 L triiodothyronine in human plasma.

The theoretical and practical considerations involved in the use of ^{131}I labelled plasma proteins have been discussed in a recent review by McFarlane (1957*a* 1957*b*)

7 Sulphur

Rapid excretion of labelled sulphate by man was reported by Borsook in 1937 Dziewiatkowski (19) studied sulphate excretion and distribution, using rats 95 per cent of the dose was excreted in urine and faeces in 120 hr Activity increased up to the 8th hour in bone and up to the 24th hour in bone marrow in contrast to the rapid fall in activity in blood and liver Recently the localization and the nature of retained sulphate has been examined in detail especially by autoradiography Bostrom and Jorpes (1954) summarize results of a systematic study of sulphate fixation in normal tissues of rats and rabbits High activity was found in cartilages the epithelial layer of the gastro-intestinal tract, aorta heart valves cornea, sclera of the eye and in mast cells Labelled chondroitin sulphate was isolated

A considerable amount of the sulphate incorporated into cartilage was considered by Davies and Y (1954) to exist in some form other than free chondroitin sulphate These workers showed activity to be highest in the zone of cell columns of epiphyseal cartilage especially in young rats

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^{35}S is a particularly suitable label for compounds which are characterized by the presence of sulphur e g methionine cystine taurine and thiamin. Labelled proteins of specific activity sufficiently high for further study have been prepared from animals to which ^{35}S labelled yeast protein has been administered (Schultze and Maurer 1957)

V INVESTIGATIONS OF THE METABOLISM OF MAJOR ANIMAL NUTRIENTS USING ISOTOPES OF HYDROGEN, CARBON AND NITROGEN

1 Carbohydrates

(a) *Absorption*

Atkinson Parsons and Smyth (1957) studied the absorption of [¹⁴C]glucose from intestinal loops of dogs They point out the difficulties involved in investigations of glucose absorption by chemical analysis it is necessary to introduce glucose solutions of high concentration into the lumen if differences between arterial and portal blood are to be measurable and furthermore it is by no means certain that all the glucose in portal blood has come from the lumen Atkinson *et al* (1957) found that 70–80 per cent of the absorbed glucose appeared as such in mesenteric venous blood 7–17 per cent as lactic acid and only insignificant amounts as carbon dioxide alanine and pyruvic acid Glucose was absorbed against a concentration gradient also absorption involved only a unidirectional movement of the glucose since the specific activity of glucose in the lumen did not decrease during absorption

The immediate fate of absorbed fructose in rats and guinea pigs was compared by Kiyasu and Chaikoff (1957) Guinea pigs converted about 70 per cent of the fructose to glucose in rats as much as 60 per cent of the activity in portal plasma was in the form of lactate most of which was formed in the intestine prior to absorption Only about 10 per cent of the absorbed activity was recovered as glucose from rats and up to 30 per cent occurred in unidentified compounds

(b) *Glucose Oxidation*

Wyshak and Chaikoff (1953) have studied the impaired carbohydrate utilization by liver slices from animals fasted 48 and 72 hr Oxidation of glucose to CO_2 was depressed by as much as 50 per cent but the amount of $^{14}CO_2$ obtained from fructose was not appreciably altered indicating that the defect in glucose utilization occurred at the stage of the initial phosphorylation reaction Lipogenesis from both glucose and fructose was impaired by fasting

Glucose oxidation involving reactions other than those of the Embden–Meyerhof pathway are known to occur in some micro organisms Wood (1955) reviewed their significance and discussed the possibilities of differentiating different pathways of glucose utilization in higher animals Murphy and Muntz (1957) found that 56 per cent of labelled glucose in blood perfused through rat liver was oxidized via the phosphogluconate pathway but they point out that only oxidative reactions were followed in their experiments and that other synthetic reactions could have taken place Marks and Feigelsen (1957) found that pentosephosphate reactions were more important in livers of fasting animals The relatively minor role of glucose in the energy metabolism of lactating cows was demonstrated by Baxter, Kleiber and Black (1955) Only about 10 per cent of the CO_2 exhaled was obtained from glucose

(c) *Lactose Synthesis*

Pazur and Tipton (1957) found that intact galactose and glucose were used for lactose formation by guinea pigs The mechanism of lactose synthesis *in vitro* by cows and a goat was investigated by Schambye Wood and Kleiber (1957) The glucose moiety appeared to be derived from free glucose and the galactose from hexose phosphate esters The complex distribution of activity in galactose following administration of ¹⁴C labelled bicarbonate and acetate was considered to result from the synthesis of this moiety in the mammary gland Duncombe (1957) demonstrated net synthesis of lactose from [¹⁴C]glucose by mammary gland slices of guinea pigs rats and sheep using a paper chromatography technique The glucose and galactose from rat lactose were both radioactive No active lactose was detected following incubation with [¹⁴C]pyruvate

(d) *Glycogen Synthesis*

Labelled glucose appeared to be incorporated intact into muscle glycogen in both fed and fasted rats (Marks and Hyelem 1957) Stetten and Stetten (1954) from a study of the nature of glycogen regeneration in rats established the metabolic heterogeneity of the glycogen molecule The distribution of [¹⁴C]glucose in liver and carcass glycogen was investigated by exhaustive β amylase degradation in this way maltose was removed from the unbranched peripheral tier of glucose units leaving a dextrin core Activity was found mainly in the peripheral region at first but an increasing fraction was found later in the dextrin core by the 12th hour dextrin activity was higher than peripheral activity in glycogen from liver but in samples of glycogen from carcass most of the activity was in the peripheral region Fasting had little influence on the incorporation of glucose into carcass glycogen in contrast to the marked effect on liver glycogen where activity became nearly evenly distributed 3 hours after injection of [¹⁴C]glucose The doubtful meaning of half lives of glycogen molecules and the difficulty of defining their turnover are discussed

2 Fats

The first exploratory biological experiments with tracers described by Schoenheimer and Rittenberg

of the specific activity of phosphate secreted into the intestinal tract

Weidmann (1956) studied the rate of renewal of phosphate in the long bones of experimental animals The different rates found for different species and the time relations of phosphate uptake did not appear to be explicable on the basis of ion exchange at the surfaces of crystals

A recent example of nutritional interest of the very numerous investigations of the kinetics of organic phosphate compounds is the work of Shore Zilversmit and Ackerman (1955) on phospholipid synthesis in experimental atherosclerosis in rabbits It was found that at least 90 per cent of the phospholipid appearing in the aorta was synthesized *in situ* and not taken from the plasma

Dawson (1957) points out that whereas the formulations of Zilversmit Entenman and Fisher (1943) for the calculation of phospholipid turnover in animal tissues have very often been applied the experimental conditions have hitherto never satisfied the theoretical criteria The chief reasons given for this were that the immediate precursors of the compounds under investigation were not known and that available analytical techniques were not adequate It is therefore difficult to assess the degree of approximation of estimations of the turnover of individual phospholipids in tissues However, rapid turnover is clearly indicated and Dawson (1957) discusses their dynamic biochemistry which was initiated by the work of Hahn and Hevesy (1937) on lecithin synthesis in brain tissue of adult animals

Hevesy (1948) has reviewed studies of nucleic acid turnover in rats especially high rates were found in tissues of young rats and in the intestinal mucosa of mature animals

6 Iodine

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1939(*a* *b*) ^{15}N tyrosine was added to the diet of an adult rat the original aim being to find whether all the ^{15}N could be recovered from the urine within a short time after feeding the labelled diet which would indicate that urine nitrogen was derived directly from food nitrogen or whether some of the ^{15}N could not be recovered from urine which would indicate that nitrogen was deposited in tissues from which an equivalent amount of nitrogen had been released The amount of ^{15}N retained in the 10-day experimental period was equal to nearly half of the amount eaten by the animal so that further investigation of its distribution was possible Only 25–30 per cent of the retained nitrogen occurred in tyrosine The isotope was also found in the monocarboxylic acids histidine arginine and protein amide nitrogen Later work showed a similar high degree of incorporation of ^{15}N from leucine glycine aspartic acid tyrosine lysine and ammonium citrate (Schoenheimer 1942 Schoenheimer and Rittenberg 1940) Pure samples of all the above mentioned amino acids were later isolated from proteins of rats which had been fed ^{15}N labelled L(−) leucine and glycine The isotope content was highest in that amino acid from the protein corresponding to the labelled amino acid fed however except for lysine all other amino acids contained significant amounts of isotope

The process of reversible deamination is the mechanism of the general distribution of α $^{15}NH_2$ from amino acids but it has been shown that this process does not occur in the cases of lysine (Weissmann and Schoenheimer 1941) and threonine (Elliot and Neuberger 1950 Meltzer and Sprinson 1952)

Swick Buchanan and Makao (1953) investigated the normal amount of fixed carbon in amino acids Rats were exposed for 12 to 85 days to a constant low partial pressure of $^{14}CO_2$ Activity of liver carbon became constant after 10 days but that of muscle was still increasing after 85 days The relative activity of the carbon atoms of various amino acids at or close to the position of equilibrium gave a measurement of the normal relation of atoms of certain amino acid carbon chains to those of metabolites involved in carbon fixation The results obtained were in general consistent with the operation of known metabolic reactions 25 per cent of the carbon from both carboxyl groups of aspartic acid and from the α-carboxyl group of glutamic acid represented fixed carbon This was consistent with the relation of these groups to oxaloacetic and α ketoglutaric acids With the exception of threonine and methionine there was almost no radioactivity in the essential amino acids The small incorporation into arginine and proline suggested that although it is known that there is a possible linkage of amino acids with glutamic acid interconversion of the carbon chain had not taken place to any marked extent The guanidino group carbon of liver arginine was nearly all derived from CO_2 Fixed carbon in arginine glutamic acid aspartic acid and alanine from liver was almost twice as high as values obtained for the amount present in these amino acids isolated from muscle Apparently mixing of amino acids between these tissues had been far from complete Recently Swick and Handa (1956) reported further work on the distribution of fixed carbon in amino acids as affected by the presence and absence of certain non-essential amino acids in the diet Fixed carbon was two to three times as high in proline arginine glutamic and aspartic acids when these amino acids were omitted from the diet The extent to which these results may indicate an increased utilization of fixed carbon was discussed

Hogstrom (1953) fed $^{13}CH_3{}^{14}COONa$ to partially hepatectomized rats and investigated the isotope distribution in fourteen amino acids isolated from proteins of regenerated liver Here also the results were in general accordance with the known biochemistry of the amino acids and their precursors Glutamic acid aspartic acid alanine serine glycine proline and arginine were labelled The essential amino acids contained only insignificant amounts of isotope It was concluded that ^{13}C and ^{14}C distribution in the labelled amino acids was in accordance with Krebs cycle reactions

(b) *The Time Relations of Food and Body Proteins*

The question of the interrelationship of body proteins was already being discussed at the time of the introduction of isotope methods of investigation Holman Mahoney and Whipple (1934) wrote of the dynamic equilibrium of plasma and tissue proteins Borsook and Keighly (1935) developed a theory of continuing metabolism of nitrogen in animals Part of their evidence was the observation by Martin and Robison (1922) that there was a logarithmic decrease in nitrogen excretion when individuals on a high protein diet had changed to a low protein diet This according to the theory of Borsook and Keighly could indicate that body protein was being degraded in the manner of a first order reaction It was postulated that such degradation was in continuous operation being balanced by storage of an equivalent amount of exogenous nitrogen when animals were in positive nitrogen balance The direct experimental attack on this problem which appeared to be excluded was made possible to some extent by the use of isotopes

The ^{15}N-content of urea excreted when animals in positive nitrogen balance were fed labelled amino acids was equal to a relatively small part of the isotope injected Since so much of the urea nitrogen was the product of body protein degradation it followed that urea nitrogen could not be a measure of exogenous

(1935) were connected with fat metabolism Mice were fed diets containing different levels of deuterated fats The distribution of the isotope among the fat from the depots and internal organs and the amount of deuterium in the body fluids at the end of the experimental period were determined Feeding was restricted so that the animals lost weight but in spite of this a large proportion of the absorbed fat was found in the depots The procedures employed were adequate for dealing with the small samples obtained even from animals fed 1 per cent labelled fat in their diet Mice fed 4 days on a 1 per cent labelled fat diet were found to have deuterium in the depot fats equivalent to 47 per cent of the deuterium of the fat ingested their weight loss was 5 9 per cent Body fluids contained heavy water equivalent to 20 per cent of the D in the ingested fat

Further fundamental knowledge of the dynamics of fat metabolism was obtained by another experimental procedure again with deuterium (Schoenheimer and Rittenberg 1936 Rittenberg and Schoenheimer 1937) Animals were given water containing a constant atom per cent excess of deuterium it was shown that the amount of isotope in their body fluids could be held fairly constant in this way Fats synthesized during experimental periods were found to contain firmly bound deuterium Since the isotope content of hydrogen from the unsaturated fat (and cholesterol) eventually approached one half the isotope content of hydrogen in the body water it appeared that the labelled lipids had been synthesized from a small possibly two-carbon unit This unit was later shown to be acetate

The steps involved in the biosynthesis of fatty acids can now be studied in detail in cell free preparations Hele Popjak and Lauryssens (1957) described a partially purified enzyme system from rat mammary glands by which the synthesis of fatty acids from acetate is achieved in the presence of coenzyme A adenosine triphosphate and stoichiometric amounts of reduced diphosphopyridine nucleotide

It has been possible to follow directly the fate of absorbed fat containing [^{14}C]palmitic acid by the techniques of Bollman *et al* (1948) for the cannulation of lymphatic ducts French and Morris (1957) studied the rate of removal of chylomicron fat from the circulation of labelled rats Chyle was collected from a donor animal glyceryl tri [1 ^{14}C]palmitate being administered through a gastrotomy tube 0 2 to 2 0 ml amounts of the chyle were injected intravenously into the experimental animals The activity in the circulation decreased rapidly and exponentially The rate of removal was inversely proportional to the amount of fat injected The rate of removal following injection of 0 2 ml of chyle had a half life of 6 2 min, having probably a closer measure of the circulatory half life of chylomicrons under physiological conditions The curves showing the disappearance of activity indicated that components were being removed at different rates When the chyle particles were fractionated by centrifugation and injected separately it was found that the fraction of visible chylomicrons disappeared more rapidly than the invisible particles Some chylomicrons were found to enter lymph following intravenous injection Morris (1958) has also studied the rate of oxidation of chylomicron fat by the rat under physiological conditions The material was readily available as shown by the sharp rise in $^{14}CO_2$ exhaled to a maximum between one and two hours The rate of oxidation was increased by starvation but the percentage oxidized was inversely related to the amount of fat injected Only oxidation of fat was measured and the amount of fat entering other metabolic processes was probably increased when the amount injected was increased Glucose or olive oil given intravenously or by mouth in isocaloric amounts caused similar reductions of the rate of oxidation of labelled fat

The contribution of dietary stearic acid to the milk fat of rats was investigated by Glascock, Duncombe and Reinus (1956) using tritium labelled stearic acid Activity was detected in the milk fat after 4 hours reached a maximum within 24 hours and was measured for up to 37 days 54 per cent of the administered activity could be recovered in the milk fat The results suggest that dietary fat contributed not more than 25 per cent to the milk fat and that the short-chain acids of the milk fat did not arise mainly by the degradation of long-chain acids

The question of the mechanism of the conversion of fatty acids to carbohydrate has been examined by Weinman Strisower and Chaikoff (1957) Applications of isotopes in investigations of the role of the Krebs cycle as the synthetic pathway are described It is pointed out that demonstration of the incorporation of isotope into glucose from a labelled fatty acid does not necessarily mean that net synthesis has occurred The main pathway by which incorporation occurs is via acetyl coenzyme A but net synthesis only takes place if there is an influx of other metabolites into the Krebs cycle. The interdependence of the processes is measurable for example by observing the ratio of $^{14}CO_2$ recovered from [1 ^{14}C]acetate to that recovered from [2 ^{14}C]acetate

3 PROTEINS

(a) *Some General Metabolic Inter relationships of Amino Acids Indicated by Isotope Investigations*

The first account of an experiment on the fate of a ^{15}N labelled amino acid added to a normal diet was given by Schoenheimer Ratner and Rittenberg in

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protein metabolism according to Folin s theory (Folin 1905) It seemed rather that body proteins were in dynamic equilibrium with a ' pool of free amino acids derived from both the digestion of food proteins and the degradation of body proteins Schoenheimer Ratner and Rittenberg (1939a) concluded that a relatively large amount of reactive tissue nitrogen had been shown to be in equilibrium with dietary nitrogen It had been found that some body constituents such as glutamic and aspartic acids and some proteins of liver serum and other organs were more actively involved in the general metabolic mixing Although muscle and skin proteins were less active on the basis of unit weight, most of the total nitrogen replacement occurred in those tissues (Schoenheimer Ratner and Rittenberg 1939b)

Later work on this question has included investigations involving longer experimental periods and examination of more purified protein samples It has been reported that a high proportion of body proteins are relatively stable in the adult Erythrocyte protein of dogs was found to be stable subsequent to formation of the mature cells (Bale *et al* (1949) Grinstein Kamen and Moore (1949) reached the same conclusion with regard to the globin of intact erythrocytes of dogs and a rat Kruh *et al* (1957) found that the specific activity of glycine from mature rabbit erythrocyte proteins was not constant Collagen protein which was estimated to equal about one third of the total mass of body protein was shown to be almost completely metabolically inert (Neuberger Perrone and Slack 1951) Myosin synthesized by rats from ^{14}C glycine was shown to be stable for about thirty days (Dreyfus Shapira and Kruh 1956)

Olesen Heilskov and Shonheyder (1954) have analysed the rate of excretion of ^{15}N by three subjects during 360 hr periods following single doses of ^{15}N glycine It was considered possible in this way to obtain information about the size of certain nitrogen pools and the rate of synthesis of proteins associated with the pools—a pool being defined as a group of substances subject to continuous exchange of nitrogen The calculations were stated to be more direct than those associated with procedures used earlier (Sprinson and Rittenberg 1949 San Pietro and Rittenberg 1953) Over the period studied Olesen *et al* found that the rate of change of ^{15}N abundance in urine could be represented by equations containing exponential functions This led to interpretati[on of] the results on the basis of a system of three [...] pools Biological considerations permitted a redu[ction] of the parameters relating to the interchang[e of] nitrogenous substances between these pools to two determinable systems or models The three [pools] were associated with a blind pool wher[e the] amount of ^{15}N leaving the pool in the experimen[t was] a negligible fraction of the total nitrogen in the [pool] The blind pool (V) may represent the more s[table] proteins of muscle skin and connective tissue [and] body components probably corresponding to [...] more active pools were—Pool V, nitrogen co[ntent] 8 to 12 g mostly urea Pool P nitrogen of a[mino] acids which were in equilibrium with glycine nitr[ogen] nitrogen content 1 5 to 2 1 g Pool Q the more l[abile] body proteins half lives 3 6 to 6 0 days nitr[ogen] content on the basis of one model 84 to 162 g o[n] the basis of the other model 274 and 295 g [...] second model would be more suitable where a [con]siderable proportion of muscle proteins were m[eta]bolically active to the same extent as plasma prot[eins]

Estimates of half lives of various plasma protei[ns] determined by isotope and biological methods [have] been summarized by Neuberger (1957)

The relative stability of different body constitu[ents] of rats was investigated by Thompson and Ba[...] (1956) and the experimental periods represente[d a] significant fraction of the life span of the anim[als] Adult rats were given drinking water labelled [at a] constant level with tritium for a period of four mon[ths] a second group of animals were exposed to triti[um] labelled water from conception to an age of six mon[ths] Body components would thus incorporate and rel[ease] tritium according to the rates of their synthesis [and] degradation during the experimental period The r[ate] of change of activity in different organs and comp[on]ents were determined after removal of the label[led] drinking water Components with half lives of [the] order of a few days were distinguished only in li[ver] lung stomach and intestine It could be conclu[ded] that approximately one half of the total orga[nic] material of the rats was degraded and resynthesi[zed] with apparent biological half lives longer than [...] days Most of the collagen of mature rats appeared [to] be metabolically inert

VI TRITIUM

1 The Use of Tritium as a Label

Tritium as a tracer for hydrogen and as a means of labelling other molecules offers very great advantages It is available carrier free at a specific activity of 2 6 curies per standard ml at very low cost The 12 5 years half life is sufficiently long to make correction for decay unnecessary during most biological experimen[ts] The energy of the β radiation is very low (maximu[m] 18 Kv) Thus no radiation shielding is necessary ev[en] for handling curie quantities also much higher do[ses] of tritium can be administered to experimental anim[als] than of any other isotope without fear of toxic effec[ts]

The low energy of the β particles imposes some restriction on the types of assay method which can be used but in fact this is no disadvantage since the methods which are applicable include all those of the gas phase assay type which are inherently more sensitive and reproducible than any others

A further and very important advantage is the possibility of synthesis of labelled compounds by hydrogenation and when suitable precursors can be made there need only be a single hot stage Examples are given below of the synthesis of labelled amino acids involving very little manipulation of highly active material

The use of tritium as an indicator for hydrogen has been criticized on the basis of the magnitude of the isotope effects involved Thompson (1954) and Thompson and Ballou (1956) have discussed this aspect in detail and conclude that notwithstanding isotope effects valid comparisons can be made of rates of incorporation of hydrogen from water into body constituents The use of tritium as a label for water is discussed in Section III 1 The isotope effect found by Anbar and Lewitus (1958) would be of importance in the determination of rates of turnover of body water but not in measurements of total body water or rates of equilibration In the case of compounds labelled with tritium the possibility of an isotope effect must be considered for any particular application Thompson and Ballou (1954) found that the tritium deuterium ratio remained constant in all tissues following incorporation from water containing tritium and deuterium Thus the rate of degradation of labelled body constituents was not influenced by an isotope effect Biggs and Kritchevsky (1952) and Hellman *et al* (1955) studied the metabolic behaviour *in vivo* of ^{14}carbon and tritium labelled cholesterol and found no significant differences

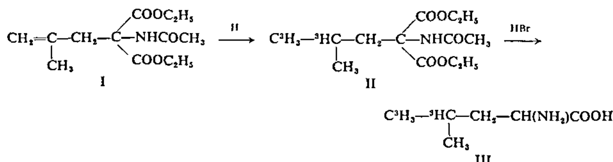
2 The Synthesis of Tritium labelled Leucine and Lysine

Special procedures are usually necessary for the synthesis of metabolites containing radioactive isotopes The two chief reasons for this are—(1) At this stage radiation hazard is likely to be considerable (2) it is necessary to ensure that the specific activity of the final product is as close as possible to that of the reactant which initially contains the radioactive isotope It follows that favourable methods should involve the minimum of manipulation of radioactive material and the minimum number of reactions involving isotopic material

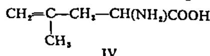
Theoretically satisfactory procedures for the synthesis of tritium labelled amino acids may be based on a single reaction in which unsaturated analogues are hydrogenated with tritium-containing hydrogen Tritium labelled leucine and lysine both of which are particularly useful for the preparation of labelled proteins have been synthesized in this way from available unsaturated analogues

(a) *The Synthesis of* DL [^3H]*leucine*

Method 1 This method based on the method of synthesis of DL leucine of Albertson and Archer (1945) has already been described (Done and Payne 1956) The relevant reactions are the reduction of ethyl 2 acetamido 2 carbethoxy-4 methyl-4 pentenoate (I) with tritium containing hydrogen in the presence of Adams catalyst followed by hydrolysis of the tritium labelled ethyl 2 acetamido 2-carbethoxy 4 methylpentanoate (II) to labelled leucine (III)



Method 2 The first method is not ideal in the sense that two reactions are involved Goering Cristol and Dittmer (1948) described the preparation of an unsaturated leucine analogue β methallylglycine (IV) and



we have prepared labelled leucine by hydrogenation of this compound It was convenient to use an aqueous solution of the free unsaturated amino acid in spite of exchange of tritium with water and labile amino and carboxyl group hydrogen Preliminary experiments had shown that reduction of the double bond occurred at a much greater rate than the exchange reactions and that labile hydrogen could be removed easily from the leucine by alternate solution of the product

in water followed by evaporation to dryness in a vacuum desiccator This second method was effective for the preparation of a 477 mg sample of leucine containing 75 μc/mg, the sample prepared by method 1 contained 3 35 μc/mg

(b) *The Synthesis of* DL [³*H*]*lysine*

We are indebted to Prof R A Raphael for an early account of the synthesis of an acetylenic lysine analogue, 1 5-diamino-1-carboxybut 3 yne (V) (Dobson and Raphael forthcoming publication)

DL [³H]lysine has been prepared from V by a hydrogenation procedure similar to that described for leucine, method 2 A small batch (18 mg) of the analogue gave 16 mg of labelled lysine specific activity 46 4 μc/mg The radiochemical purity of the product was established by co-crystallization with authentic lysine monopicrate recovered activity being 101 per cent of the theoretical

3 The Assay of Radioactive Isotopes with Special Reference to Tritium

In comparing assay methods for any isotope the first requirements are of sensitivity and accuracy also, when labelled compounds are involved it is in general necessary to measure the specific activity of the tracer element itself

(a) *Sensitivity*

Factors influencing the sensitivity of an assay method are—

(i) Absorption of the radiation by the sample itself and by the walls of the radiation detector

(ii) The efficiency of the radiation detector for the rays emitted

(iii) The magnitude and stability of the background level of the detector

In the case of soft β-emitters (i) is the most important factor and it is desirable to make use of an internal sample technique such as gas counting or liquid scintillation counting Since the efficiency for β rays of Geiger and proportional counters is very high the internal gas-counting technique offers the highest possible sensitivity

In the case of γ-emitters (i) is of no importance but (ii) becomes the decisive factor and the well type scintillation counter offers the highest efficiency

In all cases the background level attained is an indication of the lowest counting rates which can be accurately measured It should be noted that both proportional counters and scintillation counters afford some degree of discrimination between sample and background counts

(b) *Accuracy*

Factors influencing the accuracy of counting methods are—

(i) The stability of the counting apparatus

(ii) In solid sample counting (*a*) variations in self absorption and scattering, (*b*) variations in the geometry of the counter and sample system

(iii) At the lower levels, the stability of the background

In general the proportional counter has the advantage of very high stability and low and constant background levels (Borkowski 1949 Bernstein and Ballentine 1950 Robinson 1951) and is probably capable of better reproducibility than any other detector available It has the additional advantages of good linearity over a very wide range of counting rates and individual counting tubes have a very long and perhaps infinite life

The factors under (ii) limit the usefulness of solid sample counting Thus Freedman and Hume (1950) were able to obtain results consistent to within 5–7 per cent using solid counting techniques for ¹³¹I and ⁹⁰Sr However with liquid samples reproducibilities of ±1 4 per cent were readily obtained

(c) *Sample Preparation and Specific Activity Measurement*

There is a tendency to regard as superior methods of assay for which the counting technique is simple for example solid sample counting using an end window Geiger counter However consideration must be given to the complete process of sample preparation bearing in mind the necessity for measurement of the specific activity of the labelled element for example if ¹⁴C is being used the activity per milligram of carbon may be required in a range of labelled materials If reasonable accuracy is required this implies the conversion of all samples to a standard chemical form of reasonable purity These considerations apply to both solid and liquid sample counting techniques The solid counting methods suffer from the further disadvantage that great care is required in the mounting of samples in uniform layers and the maintenance of reasonably constant sample thickness may give rise to the necessity of frequent dilutions of high specific activity samples with pure unlabelled material

Evidently there are no easy methods for isotopic analysis and the choice of a tracer for a particular problem should not be influenced by the type of assay procedures involved

(d) *Tritium Assay Methods*

As in the case of other β-emitting isotopes the first step in the determination of the specific activity

of the hydrogen in a labelled compound by any assay method must be the conversion of the sample to a standard chemical form and this process must be regarded as a part of the assay procedure In some particular cases when only one or two specific labelled compounds are of interest this may be avoided *e g* the substance to be assayed may be introduced into a liquid scintillation system or assayed by means of a gas flow counter but such procedures cannot be regarded as a basis for general methods

Scintillation counting has been regarded by many people as a very desirable basis for a routine assay procedure for tritium but as yet no satisfactory routine procedure has been described for the transfer from a wide range of labelled materials into a liquid scintillation system

The introduction of active water into a liquid scintillation system has been described (Langham *et al* 1956) and this could perhaps be combined with some suitable combustion procedure purification of the combustion water would also probably be necessary But the whole process would then be very cumbersome and time-consuming Also the ultimate sensitivity of the method seems to be limited by the low counting efficiencies obtainable (about 9 per cent) and the accuracy at low levels of activity is probably reduced by high and variable backgrounds Thus the background equivalent activity achieved is about 0 001 μc the reproducibility at this level is not stated but is about ± 3 per cent at higher levels Finally it must be remembered that this is achieved only with elaborate and costly instrumentation coincidence counting and cooling of photomultipliers and sample being essential

Ionization chamber techniques have about the same sensitivity and accuracy for tritium assay as scintillation counters a sensitivity of 0 01 μc is claimed by Fallot Aberhardt and Masson (1957) with an accuracy of ± 5 per cent for levels above 0 05 μc Wilzbach Van Dyken and Kaplan (1954) claim a B E A of 0 00025 μc with a ± 1 per cent of accuracy for levels above 0 00065 μc These levels are achieved with much less complex instrumentation than the scintillation counter and in combination with a simple method of generation of gas from labelled materials such as that described by Witzbach Kaplan and Brown (1953) represents a convenient assay method for higher levels of activity

Solid phase counting of tritium is possible using a windowless gas flow counter and efficiencies of 2–3 per cent have been reported by Eidinoff and Knoll (1950) Reproducibility is probably poor ± 15 per cent for infinite thickness samples of NH_4Cl (Jenkins 1953) Such a method can be very useful for rapid checks of activity during the preparation of high specific activity substances

By far the most sensitive methods for tritium assay are those based on gas counting and the good reproducibility inherent in this technique makes it a most desirable basis for a routine procedure Robinson (1955) has described the use of a proportional counter filled with methane generated by the action of water containing tritium on methyl magnesium iodide Glascock (1954) describes the use of *n* butyl magnesium bromide to produce tritio-butane which is used as the filling for a Geiger counter Robinson (1955) does not mention the combination of this technique with a combustion method for estimation of tritium in labelled materials Glascock (1954) describes the use of a preliminary tube combustion

A method for the assay of tritium based on the use of a proportional counter filled with the gas generated by the reaction of water containing tritium with aluminium carbide (White Campbell and Payne 1950) in conjunction with a high pressure oxygen bomb combustion technique (Payne and Done 1954) for labelled materials has been developed in these laboratories during the past eight years The advantages of using aluminium carbide are firstly the gas generated has a specific activity four times higher than that produced by reaction with Grignard reagents thus a sample of given size can be contained within a counter tube of proportionally smaller volume thus achieving higher sensitivity due to the improved sample to background ratio Secondly the reaction and subsequent performance of the counter tube is unaffected by the impurities in the water produced by a simple combustion technique Thirdly aluminium carbide is a convenient reagent to handle and store as it reacts only very slowly with atmospheric water vapour at room temperature in contrast to the *n* butyl magnesium bromide which must be freshly prepared and baked *in vacuo* before each estimation

The apparatus used for combustion and subsequent gas generation is relatively simple and inexpensive (Payne and Done 1958) so that several sets can be used simultaneously thus batches of gas samples can be prepared in numbers sufficient to keep the counting apparatus in continuous operation and the fullest possible use is made of the high cost section of the equipment

The technique of mounting samples for combustion on paper (Done and Payne 1956) automatically ensures the production of constant gas volumes from samples of widely varying size Thus there is no necessity for accurate dilution of the gas sample with inactive gas from a reservoir and solutions containing unweighable quantities of active material are as easily and accurately assayed as large samples of low specific activity solids

As a routine this procedure is as convenient and rapid as any other used for isotopic analysis and a high degree of reproducibility is readily achieved

The sensitivity is such that as little as 20 $\mu\mu$c of tritium can be measured with an accuracy of ±1 per cent High sensitivity has two important consequences—(1) 95 per cent pure tritium is readily and cheaply available so that accurate measurement is possible after dilutions of the order of 10^{13} times (2) the level of activity in hot' stages of investigations (e g synthesis of metabolites) is reduced

4 Some Applications of Routine Tritium Assay

(a) *The Equilibration and Dilution of Tritium labelled Water in Experimental Subjects*

Tritium has been used to estimate total body water in rats and in humans A dose of 0 4–0 5 μc/kg body weight is sufficient for accurate measurement and 1 5–2 μc/kg for equilibration studies The method used for preparation of samples has been described (Payne and Done 1958) and it has been shown that for a normal human subject (Done and Payne 1957) an equilibration time of 1½–2 hr is sufficient The method has been applied to the measurement of total body water and half life of tritium in the body water of a group of long distance swimmers Each was given by mouth 150 μc of tritium in 200 ml of water Urine samples were taken 2 and 3 hr later and in some cases after 4 or 5 days Table 9 2 shows the percentage body water and half life figures obtained The half life figures reflect a very high rate of loss of activity possibly due to a high fluid intake the figure obtained for a normal individual being 9–10 days

TABLE 9 2

Total Body Water and Half Life of Tritium in Body Water of Long-distance Swimmers

Body weight (kg)	Body water (per cent by weight)	Half life (days)
72 3	61 3	4 4
75 9	55 7	
79 8	59 3	
82 4	57 4	6 2
85 4	61 5	7 2
87 0	58 5	
91 2	55 2	
98 4	58 3	6 2
102 6	56 4	5 9
102 9	53 2	

Mean = 57 5
S D = 2 79

The doses involved in these estimations were very much lower than those used by other for example doses of 2–5 mc have been reported (Pinson Anderson and Lotz, 1951 Prentice *et al*, 1952, Bradley *et al* 1956 Langham *et al* 1956 Fallot *et al* 1957) Recently in collaboration with Dr V Wynn St Mary s Hospital London we have examined the tissues of a patient who had received two 2 mc doses with an interval of 13 weeks Samples taken 8 weeks after the last dose had the following specific activities—

Water	5 16 × 10^{-4} μc/mg H
Muscle	4 80 ×
Connective tissue	2 40 ×
Fat	0 82 ×
Skin	0 6 ×
Fascia	0 3 ×
Bone	0 3 ×

The tissue samples were vacuum-dried, washed with hot trichloroacetic acid and extracted with chloroform–methanol The bone sample was also demineralized with acid

(b) *The Distribution of Tritium in the Proteins of a Young Pig after Administration of [³H]-DL lysine*

The distribution of tritium in the body of a young pig after administration of synthetic tritium labelled DL lysine has been reported (Done and Payne 1958) A 9 day old piglet body weight 3 kg was maintained on artificial sow milk Two injections of [³H]-DL lysine totalling 1 05 mg (57 6 μc) were given at an interval of 7 hr Seventeen hours after the second dose the animal was anaesthetized and killed by bleeding from neck veins The dose being so small it seems unlikely that the observed distribution of activity was influenced by abnormal concentrations of lysine

(c) *Distribution of Activity*

(i) *Tissue Protein* Samples prepared from various tissues by washing with hot trichloroacetic acid and solvents (Zamecnik *et al* 1951) were assayed for tritium The results are shown in the diagram (Fig 9 1) Skin collagen was prepared according to Neuberger and Slack (1953) The distribution of activity is very similar to that found by other workers e g Miller *et al* (1949) using [¹⁴C]-DL lysine and Greenberg and Winnick (1948) using [¹⁴C] glycine The relatively higher specific activities found in brain and collagen are probably related to the use of a young animal

(ii) *Lysine from Liver Proteins* A sample of the liver protein was hydrolysed in 6N HCl for 16 hr at 105°C The HCl was removed by drying *in vacuo* in the presence of KOH The resulting hydrolysate was dissolved in water and an aliquot was assayed for tritium The specific activity was found to be 1 58 × 10^{-4} μc/mg the original liver protein being 1 75 × μc/mg hydrolysate solution were

responsible for the observed decrease in digestibility on heating and it was of interest to determine whether the absorption of lysine was reduced In order to study the effect of the heat treatment on the availability of lysine the experiment was repeated using lysine labelled pork of specific activity 3 74 × 10^{-5} μc/mg, prepared as previously described Diets containing untreated and heat treated labelled pork were prepared and fed to two groups of four rats for 10 days At the end of the experiment the nitrogen and tritium contents of the faeces were determined The results expressed as a percentage of nitrogen and tritium intake are shown in the table

TABLE 9 3

Nitrogen and Radioactivity, Expressed as a Fraction of Intake in the Faeces of Animals Fed Diets containing Untreated and Heat treated Lysine-labelled Pork

	Faecal nitrogen	Faecal radioactivity	N P U
Untreated	0 18	0 10	70 (mean of 4 determinations)
Heated	0 40	0 33	35 (mean of 2 determinations)

It may be seen that the nitrogen excreted by the animals fed the diet containing heat treated pork was higher than for those fed untreated material and that this was accompanied by an increase in the excretion of tritium Thus the fall in the value of N P U is related to a reduced nitrogen digestibility and a proportionate reduction in lysine digestibility is suggested by the increased excretion of tritium

In view of the large amount of activity excreted by the rats fed untreated pork a further experiment was undertaken to investigate the proportion of excreted activity of metabolic origin as opposed to that due to undigested protein

(e) *Recovery of Radioactivity in the Faeces of a Rat after Administration of a Single Feed of Labelled Protein*

A rat weighing 375 g previously maintained on stock diet was deprived of food during one day and then at 5 30 p m was given a pellet weighing approx 2 0 g and consisting of Allinson wholemeal flour 73 per cent labelled liver protein 25 per cent and 2 per cent of black uranium oxide UO_2 The following morning the stock diet was replaced in the cage

Faecal pellets were collected each morning and for the first two days were arranged in ascending and then descending order of uranium activity measured by placing 100-mg samples of dried powdered faeces on a 25 mm diameter planchette and counting with an end window counter G E C type G M 4 Subsequent faecal collections were pooled and the uranium specific activities determined A sample of the original powdered diet was also assayed for uranium activity

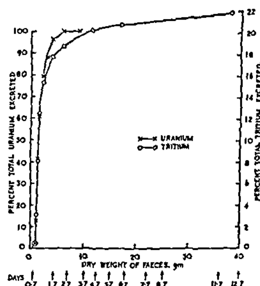


FIG 9 3

The specific activity of the tritium in the faecal samples was measured and this was continued for several days after the activity of the uranium had fallen below the limits of detectability The curves in Fig 9 3 are of the cumulative excretion of uranium and tritium percentage of total dose being plotted against grammes of faeces collected for a total period of 12 days

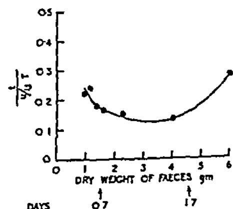


FIG 9 4

The effect of labelled material of metabolic origin is apparent from a comparison of the shapes of the

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10

The Nutritional Requirements of Embryos and the Repercussions of Deficiencies

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Contents

I INTRODUCTION

The embryo is a quickly growing organism which requires building and energy producing materials simultaneously

We shall pay particular attention in this article to the higher vertebrates—birds and above all mammals Though the facts are essentially the same in every class we shall limit our survey to higher vertebrates because of their practical interest and also because they offer the best documentation

II THE NUTRITIONAL SOURCES OF THE EMBRYO

The yolk of the egg is the fundamental source of nutrition for the lower vertebrate and the bird embryos The latter obtains the required foodstuffs previously stored in the vitellus and then in the egg albumin by absorption through the foetal membranes especially the umbilical vesicle a huge diverticulum of its intestine which contains the vitellus Some methods are now available for studying its value (Grau *et al* 1957)

The mammalian embryo takes its food from its mother and grows directly on her The supply of its foodstuffs occurs in three stages (1) the period when the blastocyst absorbs nutrients from the surrounding fluid (2) the period of absorption by the vitelline circulation in the yolk sac and (3) the fundamental period of absorption by the allantoic circulation in the placenta

In the earliest stage the ovum remains free in the Fallopian tube and then in the uterus It is a blastocyst which absorbs its foodstuffs directly from the surrounding fluid in the tube and in the uterus (Lutwak Mann 1954) Moreover its needs are very limited not only is the egg very small but it grows very little or not at all and its development has consisted mainly up till then in its subdivision into a series of small cells This stage lasts 6 days in the rat 8 days in man after fertilization In the sheep it is not until the 17th day that another source of nutrition becomes available for it is not until then that the trophoblast becomes attached to the uterine epithelium In some species this phase of liberty can be much longer In this case hormonal conditions are responsible for the late implantation of blastocysts (Mayer *et al* 1955 Neal and Harrison 1958)

When the ovum is fixed on the uterine mucosa or implanted in it it then has an easy source of nutrition and develops rapidly Before the new nutritive apparatus (the placenta) is completely organized the embryo—in a way somewhat similar to the previous stage—utilizes an embryotrophe a mixture of uterine secretion and damaged autolysing cells including blood cells

The vascular connections are provided by the allantois which fuses with the trophoblast (the outer layer of the ovum) to form the chorion The villous or labyrinthine attachments of the chorion to the uterine mucosa form the placenta This is an exchange centre between the maternal and the embryo blood by a system of two vascular networks a foetal and a maternal one in intimate contact Thus nutrients and oxygen are provided simultaneously

The structural and physiological properties of the placenta change with time The contact surfaces between the maternal and foetal blood streams become larger and larger more and more permeable So the exchanges increase during gestation Flexner *et al* (1948) studying the transfer of radioactive sodium claimed that the speed of this transfer increased with the duration of pregnancy Harrison and Silva (1956) who studied the radioactive potassium transfer concluded only that the placenta facilitates rapid exchange It must be added that for many substances the enzyme systems of the placenta play a greater role than the exchange surfaces (Wislocki and Dempsey 1946)

III THE NUTRITION OF THE EMBRYO AND THE CONSTITUTION OF THE MATERNAL NUTRITIONAL STORES

The embryo requires the materials necessary for building a new organism as well as the energy for doing so The chemical analysis of the foetus indicates the nature of the former and at least the minimum requirements for its proper development Thus estimated in absolute values these requirements will increase with the age of the embryo They will be satisfied or not as the case may be by the elements circulating in the maternal organism In keeping with this the maternal organism tends to grow richer in various reserves *i e* to store proteins mineral salts and other substances during gestation

Bar and Daunay (1905) and Murlin (1910) were the first to establish that nitrogen retention took place

during pregnancy The comprehensive investigations of Macy and Hunscher (1934) who made a careful survey of the literature and a series of continuous balances on pregnant women demonstrated the nitrogen storage and the cumulative reserves of Cl P K Na Ca and Mg We give these results in the following graphs (Figs 10 1 and 10 2) According to Kosterlitz and Campbell (1957) who recently reviewed the question not all the materials required by the foetus during the period of rapid growth are stored by the maternal body While calcium but not phosphorus is stored early during pregnancy of the rat and retention of calcium and phosphorus over the whole period of gestation is in excess of the foetal demands in the rat and in man no storage seems to occur of vitamin B_1 riboflavin pantothenic acid and biotin

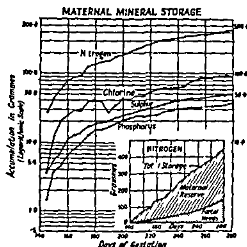


Fig 10 1 Shows the Continual Storage of Nitrogen Chlorine Sulphur and Phosphorus during the Final 145 Days of Gestation

The days are plotted arithmetically and the maternal accumulation in grammes logarithmically thus illustrating the absolute values and the relative rates of change with the progression of foetal development The inset shows the large maternal store that has been laid down in excess of the foetal needs

(*Hummel et al* 1936)

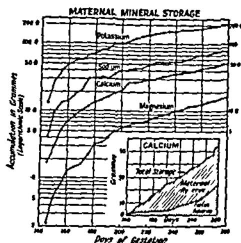


Fig 10 2 Shows the Continual Maternal Storage of Potassium Sodium Calcium and Magnesium During the Final 145 Days of Gestation

The days are plotted arithmetically and the maternal accumulation in grammes logarithmically thus illustrating not only the absolute values but the relative rates of change with the progression of foetal development The inset shows the large maternal reserve of calcium that is stored beyond the estimated needs of the foetus and the adnexa

(*Hummel et al* 1936)

Rombauts *et al* (1956) showed that a considerable retention of nitrogen may occur during the early stages of pregnancy Compared with a period immediately preceding mating the daily nitrogen retention per rat was increased by 23 mg during the period 0–4 days 19 mg during the period 4–8 days 34 mg during 8–12 days 68 mg during 12–16 days and 131 mg during 16–20 days Nitrogen was still retained on a diet in which the energy intake was unlimited but the protein intake was scarcely large enough to maintain non pregnant control rats in nitrogen equilibrium.

According to Bourdel and Jacquot (1956*a b*) these facts depend not on the embryos themselves but on the placental hormone conditions The ratio of retained nitrogen to ingested nitrogen for the same time was 0 30 when the foetuses and placenta were left intact and 0 28 when only placenta were maintained Thus the nitrogen storage by the mother is continued despite the absence of the foetus therefore it depends on the placental action

The embryo requirements become so important at the end of pregnancy that they are very often satisfied at the expense of the mother s reserves according to Jacquot (1953) This is particularly in evidence during lactation

IV THE LEVEL OF THE REQUIREMENTS

These requirements must be evaluated from three *points of view the bulk of the new living material* and its metabolic activity the absolute amounts of the nutrients used and the rates at which they are utilized

The embryo grows constantly its weight increases progressively (Fig 10 3) and it is obvious that corresponding quantities of nitrogen and other materials must be provided It is necessary to estimate the daily requirements with respect to the amounts fixed in the

building of the body these have been established by means of chemical estimation of each substance To these amounts however must be added the quantities necessary to cover the energy requirements together with the requirements of the foetal membranes and the uterus (Fig 10 4)

The example given above for nitrogen and calcium in the human foetus clearly indicates that the requirements for building the foetus are much more important in the last period of pregnancy Indeed it must be concluded that the total requirements increase daily and progressively i e with the increasing age of the foetus

On the other hand if we consider the relative increase with respect to the time unit we see that the

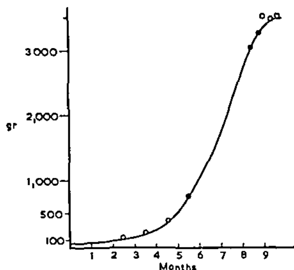


FIG 10 3 EVOLUTION OF FOETAL WEIGHT
(*Vignes* 1929)

requirements as well as the intensity of metabolism and the speed of synthesis decrease with the age of the foetus

Minot (1907) found that the rate of growth in the rabbit embryo measured by percentage increments decreased from the 9–15th day (when the rabbit adds on the average 704 per cent to its weight daily) to the 15–20th day (when the average daily addition is only 212 per cent) Minot estimated that over 98 per cent of the original power of growth in the rabbit or chick has been lost at the time of birth or hatching This is equally true in man (Fig 10 5)

The metabolic rate of the foetuses often estimated (Levine and Dann 1953) as lower than that of new born seems to be equal or higher (Fitzgerald 1958)

Kodicek and Lutwak Mann (1957) studied the fixation of vitamins B in the blastocyst of the rabbit and the young embryo They noticed especially that the level of nicotinic acid was very high in the unimplanted blastocyst In the 12th day embryo it surpassed the level of the maternal liver an organ particularly rich in this vitamin

This decrease in metabolism is well known for every animal after birth and the requirements per kilogram of animal become lower and lower with age

Lefebvres (1951) studied in the rat the effects of synthetic diets containing smaller and smaller doses of pantothenic acid Very small quantities (0–10

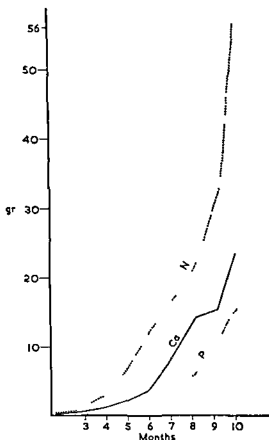


FIG 10 4 THE COMPOSITION OF THE HUMAN FOETUS AT DIFFERENT STAGES OF INTRA UTERINE LIFE
(*Hunscher et al* 1936)

mg/day) resulted in 100 per cent resorption of the embryos With an intake of 20–50 mg/day the number of resorptions decreased and abnormal foetuses were found the maximum incidence of abnormal embryos occurred with 20–25 mg/day There were no abnormalities with over 50 mg/day this amount is therefore the lower limit necessary for normal embryonic development These figures (after deduction of the maternal needs) allow an approximate evaluation per gramme of embryonic requirements These values are plotted on a descending curve which shows also

the requirements of just weaned, 6- and 10-week old rats (Fig 10 6) These values have been confirmed by several authors

The metabolic activity of the embryo is the basis of this theory to explain the preferential distribution of foodstuffs in the maternal blood stream between the foetus and maternal tissues (Fig 10 7)

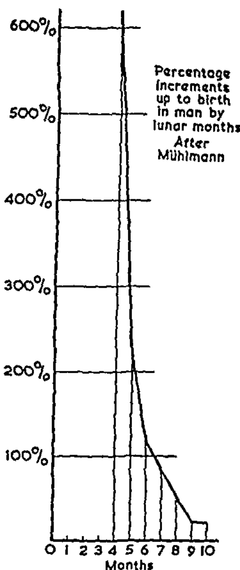


FIG 10 5 THE PERCENTAGE RATE OF GROWTH IN MAN BEFORE BIRTH

The time intervals correspond to the ten lunar months of gestation. The rate of increase in the first three months is not indicated since there are no statistical data on which to found any knowledge From the third to the fourth month the increase in growth is 600 per cent, after which it quickly drops until during the last month of pregnancy it is barely 20 per cent

(*Minot* 1907)

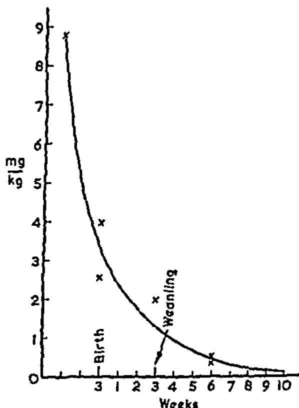


FIG 10 6 REQUIREMENTS OF THE RAT FOR PANTOTHENIC ACID AT DIFFERENT AGES

(*Lefebvres* 1951)

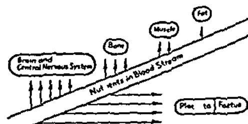


FIG 10 7 PRIORITY OF PARTITION OF NUTRIENTS ACCORDING TO METABOLIC RATE

(*Hammond* 1950)

its nutrient fixation Child (1952) suggested that the partition of nutrients in the blood stream between the different tissues was determined by the metabolic rate of each one of them the greater part being taken by the more active tissues Hammond (1944) put forward

1 UTILIZATION OF FOODSTUFFS BY THE EMBRYO

The food input is used to maintain the life of the embryo and to secure its evolution *i e* its growth and its differentiation The nutritional requirements for these various stages are not equal

Recently Spratt (1956) observed the *nutrition of* isolated chick embryos the requirements are smallest

for survival greater for differentiation and much more important for growth

According to Spratt (1952) morphogenetic processes (cell movements) require less exogenous carbohydrate nutrient than processes of cellular differentiation (histogenesis) He has suggested that there may be a *fundamental energy difference* between *form building* and tissue building activities in the embryo

The distinction between the nutritional requirements of growth and those of differentiation is well established by the information given by the culture of primordia of organs Wolff *et al* (1953) Em Wolff (1955) and Stenger Haffen (1957) adjusted a culture medium composed of amino acids mineral salts carbohydrates vitamins With this they were able to study the common needs and the special ones of various developing organs They established that a sulphur-containing amino acid cysteine or methionine was generally essential to the maintenance of life and differentiation (Fig 10 8) that arginine lysine

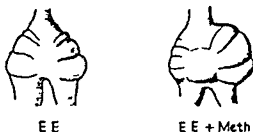


FIG 10 8 INFLUENCE OF METHIONINE ON THE DIFFERENTIATION OF THE SYRINX IN ORGANO-TYPIC CULTURE IN SYNTHETIC MEDIUM
(Em Wolff 1957)

glutamic acid together or separately were indispensable for differentiation that other amino acids and some vitamins were favourable to growth These results show that for a given organ the requirements are different for its survival differentiation or growth

Singer and Cerecedo (1957) have found that the required ratios of lysine/leucine/tryptophane are not the same for maintenance and for growth

In almost all experimental maternal deficiencies it is easier to obtain stunted abnormal embryos and the smaller deficiencies are sufficient to inhibit growth In such experiments the most frequent result is the small size of the embryo *i e* delayed growth On the other hand if we consider not the mass of the organism but its differentiation we find that even in an embryo of reduced size differentiation is normal That is at the same *age* the embryo of a deficient mother is smaller than that of a normal mother but its degree of structural achievement is about the same However if we compare the embryo of a deficient mother with an embryo of a normal mother the latter embryo being of the same weight and consequently younger we notice that the differentiation in the latter is less advanced than in the former As an example in Fig 10 9 the state of an ear in two embryos of the same weight but of different ages is demonstrated One of them has grown more slowly owing to a pantothenic acid deficiency but its differentiation is more advanced than that of the normal embryo

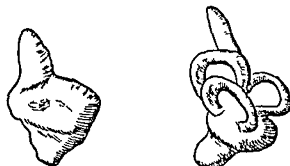


FIG 10 9 DELAYED GROWTH WITHOUT DELAYED DIFFERENTIATION
Left—ear of normal embryo *right*—ear of stunted embryo (pantothenic acid deficiency) *The latter is* older than the former but of the same size its differentiation was not as inhibited as its growth

The sensitivity of growth processes explains why growth can be modified in many ways The importance of the nutritional state of the mother and of the sharing of nutrients by several foetuses has been well established It has been demonstrated in sheep that it is easy to produce foetuses weighing less than normal by restricting the mother s diet and it has also been shown that the weight of the foetus is dependent on the number present Wallace s table (1946) demonstrated both facts very well (Table 10 1)

TABLE 10 1

***Influence of Maternal Nutrition and of the* Number of Foetuses**

(From Wallace 1946)

	Weight of the ewe (kg)	Weight of the 144-day-old foetuses (kg)	
High level diet	91 4	Single	6 07
	95 8	Twin	5 00 and 5 36
Low level diet	51 3	Twin	2 70 and 2 62
	50 4	Twin	2 77 and 2 36

Not only the quantity of food but also the appetite of the mother plays an important part in the weight of the foetus For example pregnant hypophysectomized females eat less than normal, and the weight of their foetuses is lower (Picon 1957, Knobil and Caton 1953)

The age and size of the mother also have a marked effect on the weight of the foetus It is well known

TABLE 10 2

Nutritional Requirements of Women

(National Research Council)

	Normal	Pregnancy (4th–9th month)
Calories	2 100	2 500
Protein (g)	60	85
Calcium (g)	0 8	1 5
Iron (mg)	12	15
Vitamin A (I U)	5 000	6 000
Ascorbic acid (mg)	70	100
Thiamine (mg)	1 1	1 8
Riboflavin (mg)	1 5	2 5
Nicotinic acid (mg)	1 1	1 8
Vitamin D (I U)		400–800

TABLE 10 3

Nutritional Requirements of Premature Infants

(From Levine and Dann 1953)

Water	150 cc/kg
Energy	120 cal/kg
Protein	5 g/kg
Carbohydrate	18 g/kg
Fat	2 g/kg
Calcium	160 mg/kg
Phosphorus	130 mg/kg
Vitamin A (fatty solution)	15 000 I U
Vitamin D	3 000 I U
Vitamin C	50 mg

that the weight of human new born increases with the age of the mother and the number of previous pregnancies However there is always a decrease in individual weight of the foetus in multiparous births thus in twins the individual weight is reduced

The influence of the mother's size on the nutrition of the foetus has been studied in many ways For example it has been demonstrated by crossing strains of animals of various sizes or by transfer of the same eggs into females of various sizes (Hammond 1950 Kurbatoff 1951) When Walton and Hammond

TABLE 10 4

Nutritional Requirements of the Cow and the Sow

(F A O)

	Normal	Pregnancy
	Cow 454 kg	
Calories	6 400	11 800
Protein (g)	272	544
Calcium (g)	10	22
Phosphorus (g)	10	17
	Sow 45 kg	
Calories	3 500	4 100
Protein (g)	360	410
Calcium (g)	14	16
Phosphorus (g)	9	11
Carotene (mg)	4	20
Vitamin A (I U)	2 600	13 000
Vitamin B_1 (mg)	2 5	3 0
Vitamin B_2 (mg)	3 8	3 8
Vitamin PP (mg)	12 5	12 5
Vitamin D (I U)	250 0	300 0

(1938) crossed the big shire horse and the small Shetland pony the results varied according to the size of the mother the bigger the mother the bigger the foal (Fig 10 10) Recently Hunter and colleagues



FIG 10 10 RECIPROCAL CROSSES MADE BETWEEN THE SHIRE HORSE AND SMALL SHETLAND PONY BY ARTIFICIAL INSEMINATION SHOWING THE MATERNAL EFFECT ON THE SIZE OF THE FOAL

Top line—Parents Shire stallion × Shetland mare and Shire mare × Shetland stallion *Bottom line*—Their respective foals at birth

(*Walton and Hammond* 1938)

(1954 and 1956) transferring ova of sheep into a bigger mother also obtained bigger lambs a new proof of the fact

2 GENERAL REQUIREMENTS

The definition of complete or total deficiency is not always clear In some cases such as malnutrition or limitation of nutrition in the mother at the end of

pregnancy we observe the most prejudicial effect on the weight and the general condition of the foetus both in animals and in humans

Burke and colleagues (1942) observed women with more or less deficient diets the more deficient the diet, the more numerous were the deaths of the foetuses and the overall condition (*i e* size and weight) of the new born was worsened Bourquin and Bennum (1957) observed correlations between bad dietary habits and habitual abortion Pequignot (1956) did not observe such pernicious influences in the foetuses with deficient diets but observed more abnormal pregnancies

Some figures concerning the requirements of the premature infant are interesting (Lelong 1949 Ross et 1951 Levine and Dann 1953) An average caloric intake of 110 130 calories per kg is needed 60 for basal requirement 10 for specific dynamic action 20 for the loss in the faeces and 20–30 for growth (Levine and Dann 1953)

The repercussions of the mother s general deficiency are not as important as those of specific deficiency

In various countries subsisting on a low level of food intake particularly during the last war sterility miscarriage prematurity and still births are frequent

According to C A Smith (1947) women often became sterile in Holland in 1945 The babies born of mothers underfed at the end of the pregnancy were smaller than normal ones but there was no definite evidence of an increase in abnormalities Famine in Berlin increased malformations to 1 0 per cent instead of 0 6 per cent according to Eichmann and Gesenius (1952) Nowak (1950) and Aresin and Sommer (1950) quote similar results On the other hand Ferrario and Fortina (1950) in Italy Mitani (1954) in Japan and Turpin (1955) in France found a decrease in malformations (0 74 per cent instead of 0 93 per cent) which Turpin attributes to the increase in prenatal deaths Barry (1920) claimed that starvation in the rat induces stunted new born or death and resorption of the embryos according to its severity Galeeva (1950) observed analogous results in the rabbit

Runner and Miller (1956) found in the mouse that a day without food is enough to induce abnormalities in the young The explanation of this fact remains obscure

3 Protein and Amino Acid Requirements and Importance

Proteins as well as their constituent amino acids are of primary importance

According to Weiss (1949) growth is essentially equivalent to the reproduction of existing protein molecules Their synthesis is then a fundamental process correlated with the intake of proteins or of amino acids

Burke *et al* (1943) studied the results of pregnancies among many women living on various types of diet diets poor in proteins yielded undersized and under weight new born (Table 10 5)

TABLE 10 5

Relationship between Weight and Size at Birth and the Daily Protein Content in the Mother s Diet during Gestation

(From Burke Harding and Stuart 1943)

	Proteins in grammes daily					
	Under 45	45 54	54–64	64–74	74–84	85 or more
	Weight at birth in kg					
Boys	2 948	3 175	3 374	3 628	3 770	4 139
Girls	2 665	3 119	3 402	3 516	3 657	3 856
	Height at birth in cm					
Boys	47 6	49 3	50 2	51 14	52 0	58 3
Girls	46 8	48 7	49 9	50 3	51 4	52 4

Under the same conditions Stuart (1945) noted a delayed development of bones On the contrary Pequignot (1956) did not observe such obvious disturbances For premature babies Levine and Dann (1953) claimed that in view of their heightened needs for growth 4 to 6 gm of dietary protein per kg are necessary

In the rat Guilbert and Goss (1932) observed that diets containing less than 5 per cent of proteins produce many abortions A diet without proteins produced almost entirely abortions According to Nelson and Evans (1946) the functioning of the pituitary and of the ovary is disturbed in this condition since a mixture of progesterone and oestrone was found to cure the condition

McCoy (1938) studied reproduction in the rat in five generations with various intakes of protein (15 25 and 40 per cent of casein) The data evaluated from the total weight of the litter and the individual weights at weaning indicated that the optimum content of the diet is 25 per cent of casein but 15 per cent will give satisfactory results

Conversely we must report the effects of an excess of protein Such an excess might be expected to increase the requirements of some other nutrients and to destroy the alimentary balance Aschkenasy-Lelu and Aschkenasy (1947) reported that a hyper-protein diet disturbed the oestrus cycle in the female rat Some histological modifications of the ovary and

the genital tract, accompanying this disturbance have been described by Tuchmann Duplessis and Asch kenasy Lelu (1947)

Preliminary experiments carried out on the rat show that reproduction is possible when proteins are replaced by amino acids according to McCoy (1938) In the rat amino acids required are the same as in the man arginine however is required by the rat which cannot synthesize it in sufficient quantity to secure its normal growth

In the female rat with a diet deficient in cystine from the time of fertilization or in lysine from the 8th day of gestation Lafon (1939) observed no action on the foetus The latter gets these amino acids from the mother s tissues These experiments might have given other results if the deficient diet had been given earlier, *i e* before fertilization so that the mother s tissues were already depleted in these amino acids when the experiment started McLaren (1957) observed abnormalities of the eye specially of the lens in the rat embryo from mothers receiving a poor diet (4 per cent of proteins) the proteins them selves being poor in methionine and cysteine Pike (1951) observed cataracts with a diet deficient in tryptophan Using ethionine as an antagonist to methionine Lee *et al* (1955) observed poor reproduc tive performance

Fisher (1957) studied an amino acid diet for the maintenance of egg production in chickens He established that the following amino acids were essential for this purpose arginine glutamic acid histidine lysine leucine isoleucine tryptophan phenylalanine methionine threonine and valine

Tissue culture also demonstrated that various tissues require different amino acids Using this type of technique Kieny (1956 and 1958) demonstrated the requirements for the cartilaginous tibia viz. indispens able amino acids—glutamic acid, arginine histidine lysine and methionine favourable amino acids—serine threonine hydroxyproline valine glycine alanine isoleucine ornithine and asparagine unnecessary amino acids—cysteine tyrosine, tryptophan and aspartic acid

Wolff *et al* (1953) also noted that the requirements of various organs are not the same Pigmentogenesis for example is facilitated by tyrosine and arginine (Sengel 1955) and the evolution of the neural crest of Amphibia is influenced by phenylalanine and tyrosine *in vitro* (Wilde 1955) Herrmann (1953) and Rothfelds (1954) used analogues (*i e* amino acids the structure of which is analogous to that of the normal amino acids and can therefore compete with them in metabolism) in cultures of chicken embryos With analogues of phenylalanine they induced abnormalities of the nervous system and with analogues of leucine abnormalities of the mesoblast (no somite formation)

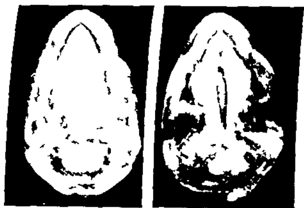
Feldman and Waddington (1955) using ethionine, an analogue of methionine disturbed the develop ment especially that of the nervous system The deficiency of definite amino acids may in fact be teratogenic More recently using other analogues of naturally occurring amino acids and purine Wadding ton and Perry (1958) induced abnormalities *in vitro* in chick embryos

The incorporation of proteins and amino acids into embryonic tissues has been studied by various authors for example the content of protein tryptophan lysine and leucine were determined at various times during the development of mouse and chick embryo by Singer *et al* (1956) and Bulbenko and Mahler (1956) found that ^{35}S labelled ovalbumin and chick serum globulin were incorporated at a higher rate than the peptides or amino acids

4 Lipid Requirements

The importance of lipids in embryonic development is linked to the presence of indispensable unsatur ated acids Female rats on a fat deficient diet usually do not ovulate and remain sterile If they should become pregnant however they have only small litters many embryos dying Burr and Burr (1930) demonstrated that this was due to the lack of unsatur ated acids and more precisely of linoleic acid Evans *et al* (1934) and Maeder (1937) confirmed these facts Guggenheim and Jurgens (1944) with such a diet given from the 13th day of pregnancy obtained normal new born but which soon lost their tails Some deficiency in vitamin B especially B_6 is probably also involved here With a diet poor in fats Martinet (1952) observed localized haemorrhages on the foetuses she demonstrated that these were prevented by linoleic acid According to Deuel *et al* (1954) the rat can become pregnant on diet without lipids but its young cannot survive Therefore the unsaturated fatty acids are necessary for reproduction and under famine conditions some accidents among humans may be the result of such a deficiency

Bernard and Bodur (1946) carried out experiments from another point of view they determined the essential fatty acids in female rats fed on a fat free diet during pregnancy and also in their young They established that the percentage of total fatty acids was lower in these adults and their young than in the controls The deficient young contained no fatty acids while the normal ones (from normally fed mothers) showed the presence of these acids So although female rats on a fat-deficient diet still had linoleic acid in their tissues they seemed unable to transmit it to their young The latter presented characteristic lesions of the tail described by Guggen heim and Jurgens (1944)

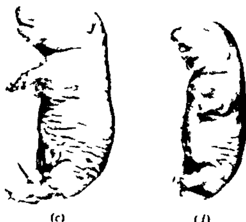


(a) (b)

(a) and (b) Riboflavin Deficiency

(a)—Control (b)—Cleft palate

(*Giroud et al*)

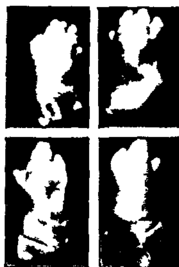


(c) (d)

(c) and (d) Riboflavin Deficiency

(c)—Control (d) Shortening of the mandible

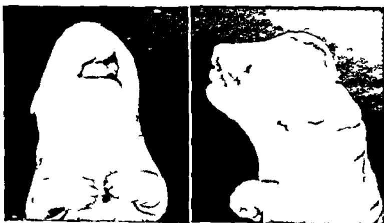
(*Giroud et al*)



(e) Riboflavin Deficiency Syndactylism

r (d) Control

((l et al)



(f) Hare lip in a Foetus of a Folic Acid deficient Mother

(*Giroud et al*)



(g) Coronal Section of a Hare lip in a Foetus of a Folic Acid-deficient Mother

(*Giroud et al*)

Plate I





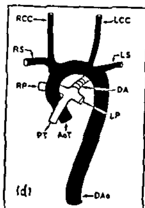
(*a*) FOLIC ACID DEFICIENCY COELOSOMIA
(*Giroud et al*)

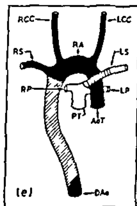


(*b*) FOLIC ACID DEFICIENCY GROSS OCULAR ABNORMALITIES
(*Giroud et al*)



(*c*) EXENCEPHALY AND GASTROSCHISIS WITH ECTOPIA OF ABDOMINAL VISCERA IN A FOETUS GIVEN PGA DEFICIENT DIET DAYS 7 TO 9
(*Nelson et al*)





(*d*) and (*e*) CONGENITAL CARDIOVASCULAR ANOMALIES INDUCED BY PGA DEFICIENT DIET
(*d*)—Definitive pattern of arteries derived from the embryonic aortic arch systems (*e*)—Right aortic arch with left subclavian artery arising from the ductus arteriosus.
(*Baird et al 1954*)

(*f*)





(*g*)

(*f*) and (*g*) PGA DEFICIENCY
(*f*) Control (*g*)—Apparent absence of right kidney with left kidney small but in normal position







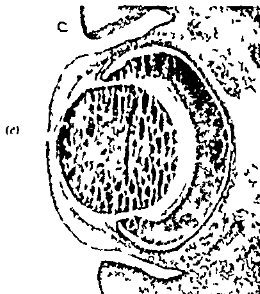


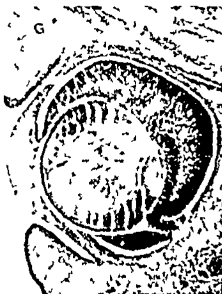
(*a*) Folic Acid Deficiency Ectopia of Viscera
(*Tuchmann Duplessis and Lefebvres Boisselot* 195[illegible])

(*b*) Pantothenic Acid Deficiency of the Mother Foetus with Exencephaly
(*Lefebvres Boisselot* 1955)

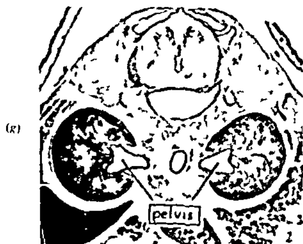
(*c*) Pantothenic Acid Deficiency Anophthalmia
(*Lefebvres Boisselot* 1955)

(*d*) Pantothenic Acid Deficiency Haemorrhages of the Extremities
(*Lefebvres Boisselot* 1955)





(*e*) and (*f*) Vitamin A Deficiency of the Mother
(*e*)—Eye of normal foetus (*f*) Eye of abnormal foetus





(*g*) and (*h*) Vitamin A Deficiency
(*g*)—Normal kidneys (*h*)—Typical horse shoe kidney Note that the renal capsul[illegible] does not separate the parenchyma of the two original kidneys ureters are not dilated and emerge on the ventro medial surface
(*Wilson and Warkany* 1947 1948)

Plate III



(a)



(b)

(*a*) and (*b*) VITAMIN D DEFICIENCY

(*a*)—Normal young (*b*)—Abnormal young of a vitamin D deficient mother bowing of radius ulna tibia and fibula broadening of distal ends of ribs

(*Warkany* 1943)



(*d*)

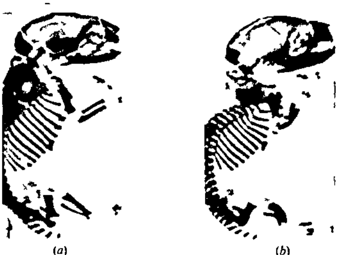


(*e*)

(*d*) and (*e*) VITAMIN A DEFICIENCY

(*d*)—Normal transitional epithelium in the urethra of a 21 day normal female foetus cytoplasm is clear and the nuclei are densely stained throughout the epithelial layer (*e*)—True keratinization on all aspects of the urethral epithelium except the most ventral in a 22 day male foetus typical cytoplasmic hyalinization keratohyalin granulation and desquamation of keratinized cells are conspicuous on the dorsal and both lateral walls

(*Wilson and Warkany* 1947 1948)



(c) VITAMIN E DEFICIENCY LITTER OF A DEFICIENT MOTHER

(*f*) and (*g*) FOLIC ACID DEFICIENCY IN VARIOUS SPECIES BY X METHYL FOLIC ACID

(*f*)—Mouse embryo with abnormal brain and gastroschisis (*Tuchmann Duplessis and Mercier Parrot* 1956 1957) (*g*)—Cat foetus with anophthalmia taillessness gastroschisis and abnormalities of the feet (*Lefebvres Boisselot and Tuchmann Duplessis* 1957)



(*f*) (*g*)

5 Carbohydrate Requirements

The importance of carbohydrates in metabolism has been well known since the time of Claude Bernard who studied this question in 1859 and demonstrated that the sugar in the rabbit foetal blood was fructose

The carbohydrates (glucose) are the first source of energy especially for the synthesis of polypeptide chains the first step of the protein synthesis (O Connor 1957)

Spratt (1956) who investigated and recently reviewed the metabolism of the early chicken embryo reached the following conclusions Carbohydrate is the main source of potential energy but not necessarily the only one during the first four days of incubation The embryo is able to utilize some carbohydrates but not others to support its continued development *in vitro* The order of activity among the utilizable substrates is—glucose = fructose galactose = maltose pyruvate = lactate

From many experiments it must be concluded that glucose can pass across the placental barrier according to Huggett and Hammond (1952) in all mammals except the Ungulata the blood sugar concentration is higher in the maternal blood than in the foetal blood The carbohydrate metabolism in the embryo is not always the same as in the adult Fructose is present in many embryos and seems to be essentially related to foetal life

According to Spratt (1956) in chick embryos explanted to carbohydrate media (glucose Ringer-buffers) containing no nitrogen sources there was little or no growth yet morphogenesis (the form building movements of cells) and cellular differentiation continued When the concentration of carbohydrate in the medium was lowered gradually or when different utilizable carbohydrates (glucose mannose fructose galactose pyruvate etc) were made available to the explanted blastoderm a whole array of differential nutrient requirements for support of the component processes was revealed Furthermore specific developmental events such as the formation of the heart brain spinal cord optic vesicles etc have both quantitatively and qualitatively different nutrient requirements For example the brain and spinal cord and optic vesicles will not develop at concentrations of sugar substrate which are quite adequate for supporting formation and pulsation of the heart The latter therefore appears to be less exacting than the central nervous system in its qualitative nutrient requirements The following scheme (Fig 10 11) demonstrates the sensitiveness of the node and of the headfold where the fore brain formation occurs

In organ culture glucose is absolutely necessary to the syrinx (Em Wolff 1957)

The nature of the carbohydrate intake of birds deserves consideration The nutritional value of the various carbohydrates depends on their nature

Medium	Stage explanted	Generalized result 20±hours *in vitro*
Ringer buffer		
Ringer buffer Glucose 10^{-3}–5×10^{-3} M		
Glucose 10^{-3} M + CH_2ICOOH 5×10^{-5} M		

Fig 10 11 Importance of Glucose for Chick Embryo Cultivated *in Vitro*
Medium without glucose with glucose and with glucose+monoiodoacetate as inhibitor
(*Spratt* 1956)

Couch Cravens Elvehjem and Halpin (1948) fed birds on diets containing different carbohydrate sources In some cases they observed a delay in the laying of eggs foetal deaths or anomalies such as syndactylia and micromelia abnormalities already noticed in cases of biotin deficiency The authors assumed that dextrin permitted synthesis of biotin in the intestine of the laying hen while sucrose and lactose did not In the new born rat Bannon *et al* (1945) noticed cataracts resulting from the presence of galactose in the mother s diet

According to Landauer (1952) the carbohydrate metabolism and its changes are very important for the developing embryo This author believes that alteration of the carbohydrate metabolism is responsible for the disturbances produced by insulin and toxins This hypothesis is confirmed by the fact that they are corrected by vitamin B treatment Troubles induced by vitamin B deficiencies may be explained in the same way

V VITAMIN REQUIREMENTS AND RESULTS OF THEIR DEFICIENCY

The knowledge of the requirements for vitamins results essentially from the observations of the various defects caused by their deficiencies which have very important and varied consequences Some do not disturb the development of the embryo on the other hand many others cause malformations more or less serious Some only delay the growth while others lead to embryonic death

1 Ascorbic Acid

The need for ascorbic acid increases during gestation and correspondingly the amount present in maternal tissues decreases (Snelling and Jackson 1939) and it is difficult to maintain the necessary level The foetus, as the placenta seems actively to fix the vitamin, and its blood is richer than the maternal blood in vitamin C Cheadle *et al* (1917) found young new born animals of scorbutic mothers had developed scorbutic symptoms Scorbutic lesions have been observed in guinea pig foetuses by Mouriquand *et al* (1935) and by Reyer *et al* (1938) We may mention here that vitamin C deficiency has been regarded as a cause of abortion, but of course such questions do not arise in the species able to synthesize ascorbic acid Premature birth is associated with lowest intake and lowest serum concentration of ascorbic acid (Martin *et al*, 1957)

2 Thiamine

The requirements of vitamin B_1 increase during pregnancy (Lockhart *et al*, 1943) Westenbrick (1943) showed that the vitamin concentration in foetal tissues increased during the last phase of gestation Ensminger *et al* (1947) noted disturbances of pregnancy in sows on a B_1 deficient diet

It is likely that thiamine deficiency does not produce malformation of the embryo in humans since there is no record of such malformations in the many cases of beriberi which have been studied In the chick embryo Naber *et al* (1954) increased the death rate by means of two anti vitamins—neopyrithiamine and oxythiamine A similar result can be obtained in the rat but no malformations were observed according to Nelson and Evans (1955*a*) The abortions occurring in the latter experiments were thought to be due to hypophyso-genital disturbances

3 Riboflavin

The influence of lack of vitamin B_2 (riboflavin) on the reproduction is well marked Disturbances due to its deficiency have been observed in birds and according to Romanoff and Bauernfeind (1942) they included great reduction of the hatching rate the limbs of the embryos were shortened (micromelia) and disturbances of the liver occurred According to Lepkovsky *et al* (1938) riboflavin deficiency is responsible for oedema anaemia dwarfism and degeneration of the Wolffian bodies Brown (1957) demonstrated the importance of transfer of riboflavin from the egg to the chick embryo

In the rat, the riboflavin deficient diet proved to have a true teratogenic effect a fact which has been established by Warkany and Schraffenberger (1944) Giroud and Boisselot (1947) and Bologna and Piccioni (1950) obtained similar results with a synthetic diet also deficient in riboflavin

The consequences of this deficiency are a function of its duration and of its intensity a long deficiency leads mainly to resorption while a very short one allows a normal development With an intermediate deficiency (started 20 to 30 days before mating and continued during gestation) there are 30 per cent of malformations among the embryos These are mainly palate and limb abnormalities Of those cases studied the general picture was as follows The whole posterior part of the palate was fissured and the nasal cavities opened into the mouth Often there was a shortening of the mandible and the incisors were underdeveloped The limbs were abnormal their distal segments shorter than those of the controls and micromelia occurred by insufficient growth of cartilage Very often the cartilaginous anlages fused together for instance in the case of the digits (syndactylia) But there were fusions of other parts especially the ribs Riboflavin determinations of the animals tissues showed that only slight deficiencies were present in such cases (Giroud *et al* 1950) (Plate I (*a*) (*b*) (*c*) (*d*) and (*e*))

Evans *et al* (1951) produced deficiencies by means of an antivitamin (galactoflavin) and obtained many malformations—abnormalities of the brain (exencephaly) of the eye (anophthalmia microphthalmia) umbilical and diaphragmatic hernia abnormalities of the large vessels and of the heart (interventricular septal defects) urogenital abnormalities (hydronephrosis ectopia of testis and ovary) Warkany (1943) had observed analogous symptoms

4 Nicotinic Acid

Human gestation greatly increases the need for niacin Lwoff *et al* (1939) observed a fall in the maternal blood content even with an adequate diet and noted no accumulation of the vitamin in the foetal tissues

In the chicken nicotinic acid presumably is synthesized during development (Dann and Handler 1941 Snell and Quarles 1941) from tryptophan (Schweigert *et al* 1948) According to Briggs *et al* (1946) a nicotinic acid deficiency in the laying hen was pro-

duced by adding a large amount of bone ossein to a purified ration creating an amino acid imbalance The birds lost weight rapidly and the egg production showed a marked drop By the third week the hatchability of the eggs declined to zero Nicotinic acid was effective in producing a recovery

Clear-cut evidence that nicotinic acid is an important metabolite was given by Ackermann and Taylor (1948) using 3 acetylpyridine as an antagonist to the vitamin These authors obtained slowed growth oedema and malformations of the feet

There does not appear to be any evidence that this vitamin deficiency produces anomalies in man No cases have been mentioned in spite of the prevalence of pellagra in many countries However Ross *et al* (1938) draw attention to the parallelism of the incidence of pellagra and that of eclampsia Experimentally Ruffo and Vescia (1941) have observed the reduction of litters in the rat on a nicotinic acid-deficient diet

5 Vitamin B_6

Experiments with laying hens involving vitamin B_6 deficiency revealed a critical need for this vitamin (*e g* there was a rapid decline in egg production) but no clear-cut evidence was obtained to show its importance in embryonic development According to Cravens (1952) the injection of desoxypyridoxine (2 4-dimethyl 3 hydroxy 5 hydroxymethylpyridine) into the egg prior to the start of incubation caused 100 per cent mortality of the embryos This inhibitory effect of the analogue was largely prevented by the simultaneous injection of 1 0 mg of pyridoxal hydrochloride These results demonstrated clearly that vitamin B_6 is highly essential for normal embryonic development Also using the chick embryo Karnofsky *et al* (1950) noted only toxic reactions to methoxypyridoxine

That vitamin B_6 deficiency may occur in human pregnancy is suggested by the fact that many authors found that vitamin B_6 was effective in the treatment of vomiting in pregnancy (Willis *et al* 1942 Weinstein *et al* 1943) There is a competition between the mother and the foetus according to Wachstein *et al* (1957)

Experiments of the present author on pyridoxine deficient rats showed that abortions were common in animals suffering from this deficiency Ross and Pike (1956) observed a deleterious influence of the vitamin B_6 deficiency on the development of the embryo Nelson and Evans (1949) obtained abortions with an analogue desoxypyridine It is possible that the abortions may have been due to a secondarily induced hypophyso genital deficiency since the administration of folliculin and progesterone restored normal gestation

6 Folic Acid

The part played by folic acid in the reproduction of birds was demonstrated by Taylor (1947) who noticed that its absence in the diet increased the mortality of the embryos especially on the 17th day and on the last day of incubation Moreover the folic acid deficiency appeared to be teratogenic in birds and mammals

In the bird Sunde and colleagues (1950) found angulations of the tibio tarsus syndactylia and parrot beak In the rat Richardson and Hogan (1946) as well as Hogan *et al* (1950) observed that with an incomplete synthetic diet there was 1 7 per cent of young with hydrocephalus and that the addition of folic acid partly suppressed this incidence Nelson and Evans (1951) found a complete resorption of embryos with a diet made deficient by incorporating an antagonist to folic acid Thiersch *et al* (1950) obtained similar results when using aminopterin as an antagonist

Giroud and Lefebvres (1951) showed the teratogenic part played by this deficiency They carried out experiments on the rat with a synthetic diet deprived of folic acid and supplemented by an intestinal antibiotic succinylsulphathiazole (5 per cent of the diet) to prevent the synthesis of folic acid by the intestinal bacteria When the deficiency was fairly severe they found resorption of the litters but with less severe deficiencies they obtained a series of malformations especially of the face *e g* hare lip and coloboma (Plate I (*f*) and (*g*)) Moreover they obtained coelosomia (no closure of the body wall) ectopia of thoracic organs (heart) or abdominal organs (liver intestine) Many abnormalities of the eyes occurred the eyes developed along such a pattern that they were not recognizable In these latter abnormalities the choroid fissure did not close and the retina was everted and was considerably folded Some malformations of the brain with hydrocephalus were observed (Plate II (*a*) (*b*))

With antivitamins the teratogenic action was also detected Using an antagonist methylfolic acid Richardson and Hogan (1946) obtained 20 per cent of hydrocephalus Nelson *et al* (1956) also observed various types of malformations in experimentally induced deficiency They used a diet without folic acid including an intestinal antibiotic succinylsulphathiazole and a folic acid antagonist X methylfolic acid This gave rise to a great variety of malformations the type of which depended on the developmental stage at which the treatment had taken place They obtained anomalies of the nervous system eyes skeleton respiratory and circulatory systems diaphragm and coelosomia There are also abnormalities of the urinary tract (Monie *et al* 1957) The circulatory system anomalies were studied by Baird *et al* (1954) (Plate II (*c*) (*d*) (*e*) (*f*) (*g*) Plate III (*a*))

Lefebvres (1951) and Tuchmann Duplessis and Mercier Parrot (1956 1957) extended these experi-

ments with X methylfolic acid to other species, the mouse and the cat In the latter in addition to abortions they found various malformations such as anophthalmia cleft palate club foot coelosomia taillessness, kidney agenesis In the mouse, malformations were obtained in the central nervous system (exencephaly) the eyes (anophthalmia and microphthalmia) and the face (hare lip) In humans it seems that the foetus actively fixes folic acid The concentration in the foetus is higher than in the mother (Baker *et al* 1958) The deficiency produced by an antagonist is teratogenic (Thiersch, 1952)

7 Pantothenic Acid

The importance of this vitamin in reproduction was shown in birds by Bauernfeind and Norris (1939) Taylor *et al* (1941) and Gillis *et al* (1948) Some analogous data were observed in the rat (Barboriak *et al* 1957)

Nelson and Evans (1955*b*) noticed that pantothenic acid deficiency produced disturbances of gestation —implantation defects resorption of the foetus or birth of reduced litters with underdeveloped young Lefebvres (1951) proved the teratogenic role of this deficiency using a completely synthetic diet without pantothenic acid, beginning 4 to 10 days before fertilization Then abnormal embryos, as well as dead or resorbed ones were found in the litters Two typical abnormalities were produced—exencephaly and anophthalmia (Plate III (*b*) and (*c*)) In the former the brain protrudes over the head, the bony vault of the skull not being developed A thin skin often exists but it is not complete the brain cavities remain open to the exterior The brain structures are irregular especially in the anterior parts prosencephalon and mesencephalon In the most severe cases the abnormalities are identical with anencephaly well known in humans and in which the brain is completely open On the borders the neural tissue is continuous with the epidermis Many other parts of the brain are necrotic These malformations are produced very early in development

In the latter anophthalmia the eye can be completely lacking without optic cup or crystalline lens In microphthalmia the eye is represented only by small residues more or less normally constituted In both cases the eye annexes are present—eyelids lachrymal glands etc Both anomalies are relatively precocious malformations

Some other anomalies were observed especially ectocardia and oedema and vascular alterations with stases at the extremities of the limbs often appeared The latter occurring comparatively late led in their turn to a destruction of the skeleton of the finger therefore to a secondary ectrodactylia (Giroud *et al* 1955) (Plate III (*d*))

Zunin and Borrone (1954) used pantoyltaurine as an antagonist to create a pantothenic acid deficiency They obtained some malformations similar to the previous ones among which were exencephaly oedema and vascular lesions With sodium ω methyl pantothenate Nelson *et al* (1957) have induced many abnormalities Recently Goetinck *et al* (1957) observed abnormalities in birds Injecting eggs with ω methylpantethine a pantothenic acid antagonist, they observed high mortality and retarded development of the brain and neural cord head structures were sometimes completely absent abnormal curvatures of the trunk hypertrophy of the gizzard micromelia curled toes and thin amuscular legs All the embryos from the treated eggs were smaller in size and weight than those from the control eggs

8 Biotin

A biotin deficiency in the hen resulted in a rapid decline of hatchability of the eggs (Cravens *et al* 1944) and the appearance of extensive gross developmental abnormalities of the embryo—parrot beak crooked tibiotarsus and short twisted tarsometatarsus occurred most frequently In addition syndactylia congenital perosis and ataxia have been observed A biotin deficiency results in two peaks of embryonic death one during the first week of embryonic development and the other during the last three days As the degree of depletion of the hen increases the time of embryonic death may gradually shift toward the early period The most critical need for this vitamin during embryonic development appears to be during the early stages of growth of the embryo

In the female rat, we noticed that biotin deficiency, created by means of succinylsulphathiazole or avidin, was not teratogenic the accidents then observed in some foetuses seemed rather to be the result of a polydeficiency With a deficient diet containing white of egg and continued for a long time William (1957) and Cooper and Brown (1958) found many resorptions of embryos and lesions of the heart liver and veins in the survivors

9 Vitamin B_{12}

In poultry Lillie *et al* (1949) noted that B_{12} deficiency disturbed reproduction Olcese *et al* (1950) and Milligan and Combs (1950) reported haemorrhages and myoatrophies in addition to an increased mortality Ferguson *et al* (1954) noticed alterations of the heart liver brain and spinal cord as well as oedema and haemorrhages

In infant rats O Dell *et al* (1951) were able to *prevent hydrocephalus from* nutritional origin by addition of vitamin B_{12} to the diet in the early stages of gestation. Jones *et al* (1955) found only lesions of the heart, liver and brain in the rat In the mouse

according to Jaffe (1956) reproduction was maintained but the further development was hampered This author also observed in the rat a high mortality an infranormal weight of the young and low tissue content of vitamin B_{12} From his results he was able to deduce the vitamin B_{12} requirements of the rat for a normal gestation Newberne and O Dell (1958) have observed hydrocephaly and Ransdell (1956) ocular abnormalities Arscott *et al* (1955) inhibited the hatching of hen eggs by injecting an antimetabolite of vitamin B_{12}

In the human the foetus actively fixes B_{12} at the expense of the mother despite the better absorption of the vitamin from her intestine (Hellegers *et al* 1957 Boger *et al* 1957 Girdwood 1957 Prystowsky *et al* 1959) The blood of the newborn is richer than that of its mother except in the case of prematurity (Dumont and Karlin 1957)

10 Para aminobenzoic Acid

Ershoff (1946) tried the action of massive doses of *p*-aminobenzoic acid or inositol (1 per cent) on the growth and reproduction of the rat He detected no adverse effects

In organ cultures besides nutritional substances (plastic or energetic) Wolff (1957) recognizes growth stimulating substances acting like catalysts he considers para aminobenzoic acid as such for the cartilaginous anlage of the tibia (Kieny 1953)

11 Carnitin

Liebecq Hutter (1956) pointed out the favourable part played by carnitin or vitamin BT in the bone development Kieny (1957) made the same claim for dicarnitin as well as for carnitin high concentrations of these growth factors are able to replace *p* amino benzoic acid

12 Choline

According to Ensminger *et al* (1947) choline deficiency caused a lack of motor co ordination and a high post natal death rate in the piglet Jukes (1940) observed a thickening of the leg bone in the chicken and the turkey

13 Vitamin A

We have known for a long time that vitamin A is necessary both to the female and to the male as proved by vaginal changes (Long and Evans 1920) and testicular injury (Mason 1933) resulting from its deficiency

In the laying hen the dietary content of vitamin A is of great importance McClymont and Hart (1948) described the effect of vitamin A deficiency on egg production, hatchability and chick viability Wagener and Harms (1943) noted the influence of vitamin A on fertilization and hatchability According to Bearse and Miller (1937) an egg containing insufficient vitamin A has reduced hatchability

In the rat Henry *et al* (1949) claimed that vitamin A was transferred to the foetus from the mother When the maternal stores were high (20 30 000 I U in the liver) the new born s liver contained 5–10 I U when the stores were low the new born s liver contained only 1 I U

Toverud and Ender (1935) demonstrated that in humans the amount of vitamin A in the new born s liver was related to the maternal ingestion during pregnancy Lewis Bodansky and Shapiro (1943) stated that vitamin A passed easily through the placenta while carotene was retained almost entirely Neuweiler (1950) observed the transfer of vitamin A through the placenta but in limited quantities

In premature babies the concentration of vitamin A is lower than in those of full term (Henley *et al* 1944) Therefore it is desirable according to Levine and Dann (1953) to administer supplements rich in vitamin A

The requirements of vitamin A during pregnancy have been estimated The daily needs of a non pregnant woman evaluated at 5 000 I U rises to 8 000 I U during pregnancy according to the Food and Nutrition Board of the National Research Council (U S A) and also to Hughes *et al* (1950)

In the sow the requirements which amount to 150 International Vitamin A Units of carotene per kg of body weight per day for growing animals reach 200 units for pregnant females The repercussions of vitamin A deficiency on reproduction were observed long ago in the sow by Norfeldt (1945) He found that the number of still born was twice as high in the deficient animals as it was in the controls Their vitality was diminished and death was frequent In the skin there was a dilatation of the superficial vessels and a stoppage of the growth of hair

The teratogenic action of the vitamin A deficiency was established more recently Hale (1935) found that a maternal deficiency in vitamin A was responsible for the occurrence of embryological accidents in developing piglets Some foetuses died and others were puny Vitamin A deficiency may cause a whole series of malformations—eye anomalies (anophthalmia and microphthalmia) hare lips cleft palates ear anomalies and fusion of both kidney anlages into one (horse shoe kidney) Confirmation is given by Goodwin and Jennings (1958)

In the calf Moore *et al* (1942) observed eye atrophy due to compression of the optic nerve at its cranial exit According to Wolbach and Bessey (1941) the bone thickening which narrows the optic foramen and causes this compression is due to only a very

slight deficiency The slightness of this deficiency was shown by Davis and Madsen (1941)

In the pregnant rat Andersen (1949) noticed that vitamin A deficiency caused diaphragmatic hernia in the embryos Warkany and Schraffenberger (1946) described a series of malformations in the young of rats deficient both in vitamin A and carotene during their pregnancy The eye malformations they noted included lack of development of the anterior chamber the iris and the ciliary processes A fibrous conjunctive tissue replaced the vitreous body and there was a coloboma *i e* the persistence of the choroidal fissure Since then Wilson and Warkany (1947 1948) have reported other anomalies first of all of the urogenital tract The kidneys showed a retarded development and remained confined in the pelvis and merged on the median line (horse shoe kidney) (see also Hale s work previously mentioned) The testicles often did not descend and both male and female vector canals persisted The male showed a tendency to develop as an intersex moreover the external genital apparatus was abnormal the urethral groove remaining open instead of closing (hypospadias) Anomalies of the cloaca of the urogenital sinus and of the Muller canals also occurred (Plate III (*e*) (*f*) (*g*) and (*h*)) These authors also observed anomalies of the large vessels and of the heart. First of all there were the most varied anomalies in the evolution of the aortic arches *e g* aortic arch on the right, two aortic arches one on the right the other on the left The heart itself presented an incomplete interventricular partition Rokkones (1955) found cases of hydrocephalus It should be pointed out that the foetus can also show deficiency lesions characteristic of the adult such as urethral keratinization

This deficiency finally leads to 75 per cent of abnormal embryos in the rat (Wilson and Warkany 1948) Jackson and Kinsey (1946) have shown its marked teratogenic action This was also found in other species particularly in the rabbit (Millen *et al* 1954)

In man Sobel *et al* (1958) claimed that mongolism would be the result of a disturbance of transfer of vitamin A from the mother to the foetus Hoet (1955) envisaged a disturbance of vitamin A in the diabetic women who give birth to abnormal infants

14 Vitamin D

Vitamin D is necessary for the normal development of the foetal skeleton Its role might even go further than that for Chavanae (1944) recorded the simultaneous occurrence of rickets and hernias in foals calves and dogs The importance of vitamin D is certainly related to the Ca and P supplies Swanson and Iob (1935) observed in the rat that even if the mother was on a diet poor in Ca and P, the foetuses received nearly normal quantities owing to the action of vitamin D This would explain the fact that female rats getting food rich in vitamin D transmit to their young some reserves which protect them against rickets, as noted by Embleton and Collings (1947) On the other hand Grant and Goettsch (1926) discovered a greater and earlier tendency to rickets in rats born from mothers receiving a diet poor in Ca and in vitamin D (Plate IV (*a*) and (*b*))

Warkany (1943) observed many skeletal malformations in about 45 per cent of the offspring of female rats that were fed a rachitogenic diet—pronounced curving of the radius ulna tibia and fibula as well as an abnormal angulation of the ribs occurred in the offspring Histologically the lesions were somewhat different from rickets Moreover, many foetuses were still born or puny

In cattle, Wallis (1938) made a comparable observation mature dairy cows were fed a diet which resulted in hypophosphataemia and hypocalcaemia they became stiff suffered spontaneous fractures and their calves had crooked legs and appeared to be rachitic.

In man vitamin D is stored in the liver of the newborn but the amounts vary greatly with the mother s diet (Toverud and Ender 1935) Rickets was detected in 5 per cent of premature children (Yllpo 1919) In some Chinese regions foetal rickets is frequent and can be prevented by administration of vitamin D (Maxwell 1934 Liu *et al* 1941)

15 Vitamin E

The importance of this vitamin for reproduction was discovered some time ago (Evans and Bishop 1922) In the chicken Adamstone (1931) observed that the deficiency caused lesions of extra-embryonic vessels leading to death in the turkey Ferguson *et al* (1954) noticed opacity of the lens and haemorrhages into the vitreous body The requirement of this bird was studied recently (Jensen and MacGinnis, 1957) In the rat Evans and Bishop (1922) showed that E deficiency caused the degeneration of placental vessels leading to embryonic death Mason (1942) and Suomalainen (1950) confirmed this Willman *et al* (1931) found new born sheep affected by muscular weakness with histological lesions and the causes of this were studied by Safford *et al* (1956) Callison and Orent Keiles (1951) found ocular alterations somewhat similar to retrolental fibroplasia in vitamin E deficient animals

Fairly recently true malformations resulting from vitamin E deficiency were obtained by Thomas and Cheng (1952) and Cheng *et al* (1957) After a long period on a deficient diet the embryo s death was just avoided by the inclusion of small amounts of vitamin E In this way they produced a whole series of malformations often associated—umbilical hernia

hydrocephalus exencephaly anomalies of the jaws cleft palate hare lip ectocardia anomalies of the tail and of the skeleton among which was syndactyly These anomalies occurred only if vitamin E was given after the 6th day of gestation and their nature varied ccording to the day of treatment It is likely that the condary ingestion of vitamin saved abnormal ibryos which would have died without it (Plate (c))

VITAMIN K

amin K deficiency may play a part in the genesis ne human foetal haemorrhages but this is still ecoatable (Plum and Dam 1940) Terado (1955) observes that the administration of vitamin K to mothers prior to delivery is effective in preventing retinal haemorrhage In the doe according to Moore *et al* (1935) vitamin K deficiency is responsible for retroplacental haemorrhages followed by abortions Brown *et al* (1947) showed that cerebral haemorrhages were found in new born rats when vitamin K and lard were absent from the mother s diet In the same conditions Hilber and Freislederer (1954) saw only myocardial lesions

Dioxycoumarin an antivitamin K induced haemorrhagic lesions and abortions in animals (Kraus *et al* 1949 Quick 1946) and in man (Sydow 1947 Tournay 1951)

VI INORGANIC DEFICIENCIES

1 CALCIUM AND PHOSPHORUS

Calcium is very important for the general development and calcification of the skeleton In the first stages of embryogenesis Ca ions are necessary (Curtis 1957) Hart *et al* (1911) observed that cows on a diet poor in calcium produced immature and stillborn foetuses This was also noted by Hart and Steenbock (1918) in the sow and by Macomber (1927) Cox and Imboden (1936) and Bodansky and Duff (1939) in the rat Tuchmann Duplessis and Mercier Parrot (1956) using a substance capable of chelating with calcium noticed interference with pregnancy and development

If the calcium content of the diet is diminished calcium and phosphorus disappear from the tissues of the mother rat (Sherman and MacLeod 1925) and additional calcium is required to prevent this both in rats and humans According to Toverud and Toverud (1931) the ossification of the human foetus is dependent on the dietary calcium and phosphorus content If it is low rarefaction of bones and teeth occurs (Booher and Hensemann 1931) vitamin D improves the condition

It is known that calcium deficient women give birth to children with altered bones as claimed by Sontag (1938) Stearns (1939) and Wake (1944) Children s teeth also depend on the mother s diet during pregnancy according to Mellanby and Coumoulos (1944) In the rat Shipley *et al* (1921) observed slender and fragile skeletons when a diet poor in calcium is fed to the mother Under the same conditions Toverud and Toverud produced hypocalcified bones in the dog Stuart (1945) and Burke (1948) claimed that there is a relationship between the calcium content of the maternal diet and the rate of ossification at birth It is a fact that a high percentage of infants whose osseous development is retarded is found in the group in which the diet was extremely deficient This retardation affects bone and tooth development

Calcium and phosphorus metabolism has been studied by Leitch (1936–7) Stearns (1939) Holmes (1944–5) and Duckworth and Warnock (1942–3) Another similar study has been carried out on women during the last half of pregnancy by Oberst and Plass (1940)

Ramsay *et al* (1938) studying the calcium concentration in the plasma noted that it decreased during pregnancy and this was confirmed by Bodansky and Duff (1941) With a concentration of less than 8 5 mg calcium per ml the diet should be considered as Ca deficient or else hypoparathyroidism must be suspected

Parallel to these changes the phosphatase concentration in the plasma was studied in pregnant women by Meranze *et al* (1937) Ramsay *et al* (1938) and Vermehren (1939) it was found that phosphatase increased during pregnancy and its concentration was the same in the mother and in the foetus

2 MAGNESIUM

The magnesium content of the maternal serum decreases during pregnancy especially in the first stages according to Wolf *et al* (1937) and the Mg content of the new born serum is higher than that of the mother according to Zaharescu Karaman *et al* (1936) Tufts and Greenberg (1937) claimed that the Mg requirements are connected with the Ca content of the diet With a diet containing 0 87 mg Ca per cent 5 mg Mg were necessary to ensure the growth of the rat Female rats fed on this diet gave birth to young of normal weight With the same Mg content and 1 16 mg Ca per cent they remained sterile If pregnancy began with a diet containing 1 66 mg per cent Ca and 13 mg per cent Mg there was abortion or the young which were born alive were immediately

eaten by their mothers Kaufmann Cosla and Tudor (1946) also studied the action of Mg on rat and chicken reproduction.

3 Sodium

Although sodium excess is harmful to the mother and probably also to the foetus (eclampsia-oedema), a minimum amount is necessary for a normal gestation as proved by experiments on the rat According to Miller (1926) gestation was normal with a diet containing 0 45 per cent of Na but the results are more satisfactory with 0 53 per cent according to Olson and St John (1925) With a very low content (0 002 per cent) Orent Keiles *et al* (1937) did not obtain normal pregnancies only two rats out of twelve became pregnant and they did not bring their litters to full term

4 Potassium

Orent Keiles and McCollum (1941) noticed disturbances in the ovarian cycle in experimental deficiency of potassium With a very low potassium content in the diet Heppel and Schmidt (1938) observed that gestation is possible in the rat but that the young were not brought up by the mothers being often eaten at birth

5 Iron

Iron is necessary for the building of haemoglobin A deficient diet maintained during successive generations induces a progressively anaemic condition in the young (Alt 1938) Adair *et al* (1943) in a study of 800 human pregnancies observed anaemia in only 12 per cent and Fullerton (1936) considered that the iron requirements of the embryo were not responsible for anaemia in the mother the anaemia of pregnancy being the result of a pre-existing deficiency Recently Bothwell *et al* (1957) claimed a withdrawal of iron at the expense of the mother whose haematopoietic activity is reduced

According to Reid and Mackintosh (1937*a* *b*) and Napier and Edwards (1941) however the viability and the strength of the new born are diminished by the mother s anaemia In a study of a large number of pregnant women Murphy (1947) noted that an unusually large proportion of the mothers of malformed offspring had received an unbalanced diet and that anaemia was considerably higher in these women

With regard to iron transfer Pommerenke (1936) noticed that 40 minutes after oral administration to the mother radioactive iron was found in the foetal circulation It passed directly to the foetus without being incorporated into the maternal haemoglobin

6 Copper

Ewes fed a diet deficient in copper may give birth to abnormal lambs (Bennets and Chapman 1937, Dunlop and Wells 1938) The new born lambs were unable to stand or to obtain milk from their dams Those that were able to get up swayed and collapsed and their hindquarters seemed paralysed thus the condition was termed 'swayback' Widespread symmetrical degeneration was found in the white matter of the spinal cord which contained cavities filled with a transparent gelatinous substance or with fluid Innes (1934) considered that the disorder seemed to be essentially a process of demyelination Instances of swayback have been observed in England Australia, South Africa Sweden and Peru Palson and Grimson (1953) have seen it when sheep in Iceland were fed with seaweed In this case the copper content of the mother s blood was one fifth of the normal and that of the lamb s liver 20–30 times lower than the normal average When pregnant ewes were given access to salt licks containing 1 per cent copper (copper sulphate) the lambs were usually free from the disease

7 Iodine

In adults iodine is necessary, perhaps essential for reproduction owing to its relation to thyroid function In foetuses iodine deficiency induced thyroid dysfunction with alterations of the skin and its appendages and, especially in human foetuses abnormal evolution of the brain As a result of this dysfunction thyroids may become hypertrophic Chemically they are found to be poor in iodine and they have the appearance of goitres

Hart and Steenbock (1918) and G E Smith (1917) noticed in countries poor in iodine that piglets have hypertrophied thyroids and a hairless skin They often produced still born or very weak young Similar abnormalities were observed in the goat (Martins 1946)

In humans the facts are somewhat different—goitres and cretinism are associated moreover the percentage of abnormalities (hydrocephaly strabismus eye defects urogenital anomalies club-feet) is increased among the populations living in the countries poor in iodine In such areas still births and neonatal deaths are more numerous than usual The new born have hypertrophied thyroids they develop cretinism very often and deafness and speech defects are almost constant According to Eggenberger (1934) of Switzerland the percentage of abnormalities is 1 45 per cent instead of 0 4 per cent which is the normal figure When the population was given iodine goitres disappeared and the percentage of abnormalities dropped to a normal level Polman (1947) and Pasma (1948) recorded similar facts in Holland In Bosnia there occurred also goitres with cretinism and

deafness and frequent appearance of abnormalities which were suppressed by a supply of iodine Many reproductive troubles were observed in cattle in the same area (Jovanic and Pantic 1957)

Lotmar (1933) studying the brain found irregularities in the development of the various cortical layers and variations of the nerve-cell distribution in iodine deficiency In some instances ectopic nests of nerve cells were found in the molecular layer of the cerebral cortex and occasionally a typical giant pyramidal cell In the cerebellar cortex ectopic Purkinje cells were found displaced into the molecular layer These histological abnormalities probably date back to foetal life

Jovanic and Pantic (1957) breeding rats on a goitrogenic soil proved the influence of iodine on reproduction the females whose diets were poor in iodine produced reduced litters while those who were given the same diet supplemented by KI in the drinking water had normal litters (46 8 per cent more embryos)

TRACE INORGANIC ELEMENTS

Traces of some inorganic elements are certainly important it is difficult to demonstrate the influence all because it is necessary to prepare very purified ts and it is hardly possible to avoid a supply of the nent reaching the foetus from the mother Others as seen below have been shown to have importance

(*a*) *Fluorine*

Fluorine can at any time act on teeth which develop late in the foetus or even after birth It has been suggested that to prevent dental caries fluorides be added to ingested food or water during the years of teeth development and of enamel calcification children of districts with water containing natural fluorides (1 2 parts/million F) have less caries than children from districts with water poor in fluorides Ast and Schlesinger (1956) describe an experiment in which Kinston (U S A) area with a water supply using 0 1 ppm F agreed to serve as control while another area Newburg (U S A) agreed to serve as the study area with its water supply supplemented to 1 0–1 2 ppm F Ten years later permanent tooth decay among children 6–9 years old was 58 per cent lower in the supplemented area No difference in bone density bone age lesions or dental fluorosis was noted Analogous data were obtained in Canada (Hutton *et al* 1956) According to Belanger *et al* (1957) fluorine would modify the fixation of calcium and sulphur

(*b*) *Manganese*

Manganese is indispensable to reproduction Daniels and Everson (1935) attributed the high mortality in young rats fed on milk supplemented with Fe and Cu to a congenital debility induced by a manganese deficiency of the maternal diet Landauer (1953*a*) as well as Lyons and Insko (1937) observed micromelia due to this deficiency Caskey and Norris (1940) obtained chickens ataxic or with micromelia when the hens were submitted to a diet very poor in manganese Gallup and Norris (1939) observed an increase in the death rate of chick embryos on manganese deficient diets they were completely developed but unable to hatch (congenital debility)

(*c*) *Zinc*

Yolk of egg contains appreciable amounts of zinc and egg production clearly involves a considerable loss of this element by laying birds Whether the metal is incorporated into the egg in order to provide a store for the growing chick or whether this method of eliminating zinc provides a channel for the hen to get rid of excess metal is at present unknown The latter possibility cannot be excluded for the hen can eliminate lead in combination with the yolk fatty acids (Bishop 1929*a* *b*) and selenium in the yolk and white (Moxon and Poley 1938) It should not be overlooked however that zinc is an essential trace element and that it will be needed in the egg by the developing chick for the production of carbonic anhydrase and possibly other zinc proteins

(*d*) *Nickel and Cobalt*

The requirements of nickel and cobalt are not known but their presence in some organs of human embryos must be mentioned (Loenau and Sakovich 1957)

(*e*) *Rhenium*

By means of labelling methods Roche *et al* (1951 1957) studied the transfer of rhenium and manganese in the hen They noted that during the period of yolk production the egg actively fixes the former much more so than the ^{54}Mn

VII IMPORTANCE OF EMBRYONIC REQUIREMENTS

The foregoing data have demonstrated that the nutritional requirements of embryos are considerable The mother either in advance as in the case of birds or progressively during the pregnancy as in mammals provides food for the embryo In the former the food is stored in the yolk in the latter the daily requirements are ensured by the placental exchanges When the supply of food in either case is insufficient

it must be called a nutritional maternal deficiency By weight the requirements of the embryo are greater than those of the young and *a fortiori* those of the adult

Some substances can be replaced by others but some cannot Any one of three unsaturated fatty acids is adequate viz. linoleic acid linolenic acid or arachidonic acid Also some amino acids may substitute for each other as demonstrated by culture of organs (Wolff *et al* 1953) In the same way studying the nutrition of chick embryonic heart fibroblasts cultivated *in vitro* Morgan and Morton (1957) subdivided the amino acids into indispensable dispensable and inhibiting In the case of various vitamins, they appear as indispensable, but for example there may be replacement of some B vitamins by a big excess of ascorbic acid However this cannot be considered as a true nutritional replacement Perhaps indispensability is an explanation of the severity of some deficiencies

VIII VARIOUS REPERCUSSIONS OF NUTRITIONAL DEFICIENCIES ON EMBRYOS

The influence of nutrition on reproduction is obvious —the favourable influence of an adequate diet seems proved the unfavourable influence of a deficient diet is undeniable In the latter case as shown above the repercussions can be very varied

1 Death or Resorption

Deficiencies can lead to embryonic death. According to the species either abortion occurs (*i e* elimination of the embryo which happens in the human) or simple resorption of the embryo takes place (*e g* in the smaller species such as the rat) This resorption may be total or partial in the latter case dead embryos being resorbed or traces of their implantation may be found, next to normal or abnormal embryos

2 Malformations

The most characteristic repercussion of deficiency on embryos is malformation Hale (1935) was the first to prove the teratogenic role of a vitamin deficiency in the sow He noticed that face mouth or eye malformations appeared in some litters The genetic origins of such accidents had been assumed for at that time there was a tendency to attribute a genetic origin to every malformation Hale disproved this hypothesis he showed that the incriminated boar had good offspring in breeding with other sows and above all that the sows themselves which had produced abnormal litters had perfectly healthy young if they received another diet Finally he showed that a vitamin A deficiency was concerned since he was able to suppress such accidents by enriching the diet in vitamin A This fundamental experiment established that a deficiency in the mother could by itself cause foetal malformations

More recently, Warkany and Schraffenberger (1944) observed a similar series of events in the rat With an imperfect diet as described above they obtained offspring with cleft palates and shortened limbs and they also established that heredity was not concerned They noticed that these accidents were avoided by adding liver or liver extracts to the diet and finally they proved that maternal deficiency in vitamin B_2 was responsible for the accidents Since then many other examples have been recorded

The malformations produced by deficiencies are very varied some are serious such as anencephaly (the destruction of the encephalon and of the skull) and coelosomia (the non-closure of the body cavity) others are relatively unimportant, such as shortening of the limbs or fusion of the ribs Between these two extremes are many malformations of varied importance The percentage of malformations varies according to the type and the intensity of the deficiency It is often 25–30 per cent it may even reach 100 per cent.

3 Stunting or Delayed Growth

One of the consequences—very frequent if not general—of a deficient diet is delayed growth This phenomenon is observed in general deficiencies (for instance in humans after famines) it is also found in various specific deficiencies principally those of vitamins This delayed growth does not necessarily involve a delay in the differentiation The foetus at birth is characterized only by a smaller size and lighter weight In all our experiments on rats we have noted this kind of delay for instance with riboflavin deficiency we obtained an average weight of only 2 39 g instead of the average normal weight of 3 05 g on the 21st day of gestation All experimenters have made similar remarks on other deficiencies which have a teratogenic effect

In his experiments on chick embryo cultures Spratt (1948) noticed that the maintenance of the embryo required a minimum nutritional intake its differentiation a larger intake and its growth a maximal intake Delayed growth may appear precociously but in general it appears more and more important near the end of gestation when there may be a complete deficiency in one or several constituents However this delay in growth probably has a more general cause for it can be observed with a number of agents, *e g* infections toxic substances or physical factors

4 Lesions

Lesions such as haemorrhages and oedemas can occur in the embryo Recently Lefebvres and Dupuis

(1956) claimed that water retention is increased in pantothenic acid deficiency Vitamin deficiencies can produce lesions in the foetus identical with those observed in adults This is the case with bones in vitamin D deficiency It is also observed in the rat with vitamin A deficiency (Wilson *et al* 1947 1953) the epithelia of the urinary system becoming keratinized This is a typical lesion of vitamin A deficiency as observed in the young or in the adult animal (Plate IV (*d*) and (*e*))

Therefore the repercussions of deficiency are numerous but only malformations are characteristic of the embryonic stage since they correspond to disturbances of the morphogenetic mechanisms specific to this period

IX EFFECTS OF A GENERAL DEFICIENCY AND OF SPECIFIC DEFICIENCY

The repercussions of a general deficiency and of a specific deficiency are very different The former are less severe

We have quoted the effects of starvation They are not as important as we might have supposed never theless starvation can lead to sterility if it begins before conception—if it begins in the later stages of pregnancy gestation is continued but under these conditions the weight of the foetus is reduced Abnormalities do not appear to be particularly common in populations living on decreased food intake The percentage of abnormalities in famines is doubled according to some authorities and dimin ished according to others This is not the case with specific deficiencies

A deficiency of protein did not induce abnormalities but the absence of specific amino acids was not so innocuous Herrmann (1953) and Waddington (1953) observed abnormal development of chick embryos in culture by suppressing some amino acids by analogues Deficiency of specific vitamins also induced abnormalities in culture moreover the nature of the abnormalities was dependent on the nature of the vitamin in question

The effects of a deficiency of a specific B vitamin are different from a deficiency of the vitamin B com plex Giroud and Lefebvres (1951) compared the results in pregnant rats of a pure pantothenic acid deficiency and of a vitamin B complex deficiency and found that abortions and especially abnormalities were less numerous in the latter It must be concluded that a specific vitamin deficiency is more severe than a vitamin complex deficiency the equilibrium of the nutrients being important

X SEVERITY OF THE EFFECTS AS A FUNCTION OF THE INTENSITY OF THE DEFICIENCY

Experimental results show that the effects on the embryo of nutritional deficiencies and especially vitamin deficiencies vary in severity That severity clearly varies with the nature of the deficiency its intensity and its duration In the case of an experi mental deficiency produced by means of antagonists the consequences will be simultaneously more sudden and more severe

In conditions of marked deficiency female experi mental animals do not have normal cycles and become sterile the same results occur in human females A lesser deficiency produces fertile females but their ova which can be fertilized do not reach their full develop ment the embryos die and become resorbed These disturbances are manifested by abnormal vaginal haemorrhages and by a decrease in the weight curve during the gestation They are finally evidenced by a reduction of the size of the litter The presence of implantation traces on the uterus confirms the disappearance of the embryos In human females abortions or births of premature infants have been observed

A lesser degree of deficiency is characterized by the appearance of anomalies The embryos develop but become malformed It has been shown that only slight deficiencies are sufficient to produce such results under experimental conditions in such conditions the deficient animals must be carefully studied during the progress of the experiment since there is a danger of obtaining either too light or too severe a deficiency

Finally deficiencies even less marked can lead to a normal development *i e* without anomalies but generally the foetuses are smaller and underdeveloped

This gradation of results is found in all cases In vitamin deficiencies in experiments in which the amount of the vitamins present in the mothers and foetuses can be chemically estimated this has been well established Giroud *et al* (1950) made this point clear as regards riboflavin deficiency comparing the liver content of riboflavin in the mother under various conditions In normal females the content was 29 5 μg per g In slightly deficient females (short term deficiencies) yielding mainly abnormal embryos the content was 19 5 μg In more deficient females (long term deficiencies) whose embryos died and were resorbed the content was only 15 8 μg Therefore there is a good parallelism between the deficiency intensity in the pregnant female and the gravity of the resulting disturbance

XI RELATIONSHIP OF THE NATURE OF THE DEFICIENCY TO THE TYPE OF MALFORMATIONS

In some types of experiment the malformations obtained seem specific for a given deficiency but not in other teratogenic experiments

In experiments using various toxic substances it is obvious that the nature of the agent used is highly important Ancel (1950) in particular clearly showed that the influence of the various toxins is not the same While some agents act on a certain process at a certain stage other agents act on different processes at other stages The various malformations which occur therefore tend to be characteristic of the toxin used

Similar facts have been observed in the case of vitamin deficiencies produced by means of a diet deprived of a specific vitamin Thus vitamin A deficiency concerns mesoderm and its derivatives resulting in disturbances mainly in the development of kidneys genital parts and vascular system and alterations of the eye particularly its mesenchymatous constituents (Wilson and Warkany 1947 1948)

A deficiency in riboflavin determines micromelias with syndactylia and cleft palates (Warkany *et al* 1942, Giroud and Boisselot 1947) A pantothenic acid deficiency mainly alters the neuraxis and its derivatives—the encephalon and the eye (Lefebvres 1951) Folic acid deficiency influences the development of the face, the eye and the closure of the body wall according to Giroud and Lefebvres (1951)

These facts thus prove that the various deficiencies have distinct influences and that each deficiency has a *specific pattern However this specificity* greatly diminishes when a deficiency is established by means of an antagonist

Nelson *et al* (1952) and also Warkany (1943) produced deficiencies of vitamin B_2 by giving galactoflavin as an antagonist They noted that abnormalities were much more varied than with the simple deficient diet They obtained not only micromelias with syndac*tylia and cleft palates but also microphthalmias* hydrocephalus hydronephrosis delayed descent of the testicles cardio-vascular anomalies diaphragmatic hernias and coelosomias

Nelson et al (1951) and Tuchmann Duplessis and Lefebvres Boisselot (1957) found similar effects with folic acid deficiency They withdrew folic acid from the diet and added an antagonist to this vitamin methyl folic acid they were able to produce quick deficiencies at various stages of embryogenesis Then they observed a whole series of anomalies—cerebral malformations cleft palates hare-lips micromelia with syndactylia club-feet and urogenital and cardiovascular malformations These anomalies obtained with methylfolic acid are not very different from those observed with galactoflavin It is probably the same in other cases in fact by means of a pantothenic acid deficiency we obtained monsters analogous to those obtained by Nelson and colleagues with a folic acid deficiency induced by an antagonist

It is worth noting therefore that the use of antagonists must not be regarded as an exact substitute for the actual absence of vitamin in the diet and that different antagonists to the same vitamin can have different actions According to Sandmann and Snell (1955) the antagonist to vitamin B_6 produces a deficiency different from the one occurring with the deficient diet According to Naber *et al* (1954), the various antagonists to vitamin B_1 do not give identical results Some actions such as that of aminopterin cannot be completely counterbalanced by the vitamin itself Cravens (1952) wisely states that antagonists cannot solve all the problems of vitamin actions Moreover it may be pointed out that the action of these antivitamins is more drastic and consequently can influence processes less sensitive than those which react to the simple withdrawal of the vitamin concerned During their experiments with antagonists Warkany (1943) as well as Runner (oral communication) noticed that anomalies obtained with smaller doses corresponded to those obtained with a deficient diet Thus according to Wilson (1954) by adding galactoflavin to a diet deficient in riboflavin there were obtained mainly skeletal malformations due to the simple deficiency, but visceral malformations were added due to the lengthening or to the intensification of the sensitive period The same fact was observed with toxins (Ancel 1950) and X rays that is the increase of the dose linked with the time factor lowers the specificity (Wilson, 1957)

Therefore it can be concluded that some specificity of the various deficiencies exists but that it is not absolute The time factor *i e* the embryonic stage at which the interference takes place is highly important

XII INFLUENCE OF THE EMBRYONIC STAGE

Dareste (1891) had already indicated how important was *the embryonic stage at which interferences* take place to induce monstrosities In his experiments on the chicken with several toxins Ancel (1950) established that the stage of embryonic evolution with which a teratogenic agent interferes is very important. This influence of time or of the stage of development, is obvious in some malformations resulting from

maternal vitamin deficiencies induced by deficient diets. As regards vitamin A deficiency in the sow, Hale (1935) noticed that if vitamin A is given only from the 30th day of gestation the litter remains abnormal. Warkany *et al.* (1942) made more precise observations in the rat deficient in vitamin B_2. If riboflavin is given only from the 14th day of gestation the embryos are malformed. On the other hand if it is given before the 13th day, the embryos are normal. The period during which the embryo is susceptible to the production of malformations of the palate and limbs is around the 13th day.

The deficiencies brought about by means of anti-vitamins also demonstrates the influence of the embryonic stage. With this technique it is possible to create a sudden deficiency at the various stages of gestation, the types of malformations are multiplied and a given type corresponds to a certain action at a determined stage.

With a folic acid deficiency supplemented by an antivitamin (methylfolic acid) Evans *et al.* (1954) observed some cerebral and cardiovascular malformations as well as coelosomias between the 7th and the 11th day. Between the 10th and the 12th day there were none of these anomalies but there were some limb malformations. With a riboflavin deficiency supplemented by an antivitamin (galactoflavin) Nelson *et al.* (1956) obtained from the 7th to the 11th day only cardiovascular anomalies, from the 7th to the 13th day there occurred many other anomalies, many of which were skeletal. Similar data have also been recorded by Warkany (1943) (Fig. 10.12).

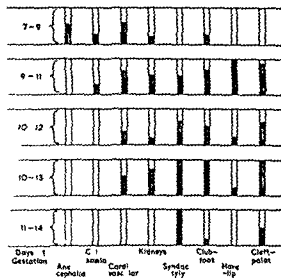


FIG. 10.12. INCIDENCE OF MACROSCOPIC ABNORMALITIES ENCOUNTERED IN ABNORMAL YOUNG OF MOTHERS SUBMITTED TO A TRANSITORY PTEROYLGLUTAMIC ACID DEFICIENCY DURING GESTATION

Black columns represent the percentage of abnormalities. The importance of the time of instituting the deficiency on incidence and on the type of abnormalities is clearly shown.

(*Nelson et al.*)

All these facts prove the importance of the time factor for the production of malformations as well as for their morphological type. This does not imply that there is no specificity of the substance as well.

XIII. MECHANISM OF THE TERATOGENIC ACTION OF DEFICIENCIES

1. DIRECT MECHANISMS

The teratogenic action may be direct or indirect, *i.e.* by way of the mother's organism.

In some cases the lack of a given nutrient may be the cause of maldevelopment. It can be assumed that the absence of some amino acids directly slows down some protein syntheses necessary to the normal building up of a particular part of the embryo (Herrmann, 1953; Waddington, 1953; Wolff, 1957).

The action of vitamin deficiencies is more complex. It is well known that many vitamins are components of enzymes. Consequently according as the vitamin intake is sufficient or not there are important variations in the enzyme activities. Thus riboflavin deficiency involves a fall of the oxygen consumption and leads to a decrease in the hepatic succinic dehydrogenase, cytochrome oxidase, *d*-amino acid oxidase, amino-oxidase and inversely to an increase in xanthine oxidase. With folic acid deficiency a decrease in choline oxidase and inversely an increase in xanthine oxidase and succinic dehydrogenase occurs (Levy, 1953).

Therefore various vitamin deficiencies lead to distinct enzymic changes with their appropriate consequences. The metabolic disturbances thus created can cause the accumulation of harmful substances or the scarcity of useful ones, thereby upsetting the cellular functions.

These disturbances can determine deficiency lesions in the foetus as well as in the adult. They were observed following a deficiency in vitamin A (Wilson *et al.*, 1947) and in vitamin D (Warkany, 1943). We noted that some late malformations arise from destructive lesions of organs and tissues already formed; an example is given by ectrodactylia following a pantothenic acid deficiency.

Some disturbances in the evolution of primordia leading to malformations can also be interpreted as disturbances of their particular physiology. Following vitamin A deficiency (Wilson and Warkany, 1947,

1948) some malformations of the genital apparatus in the male (persistence of the Muller canal hypospadias) seem to result from an insufficient endocrine secretion of the testes indeed it is well known that the morphogenesis of the genital apparatus depends on the endocrine function of the testes (Jost 1948) and that this function becomes deficient in the adult in the case of a vitamin A deficiency Therefore it can be reasonably assumed that in the embryo the endocrine function is reduced for the same reason and that the evolution of the genital anlage suffers as a consequence Disturbances of mechanisms still unknown involving the growth of cartilages and of the plates of the palate would cause micromelias and cleft palates respectively

The disturbances of the differentiation processes of a particular element *i e* the disturbances of the induction process are the starting point of the main malformations It is well known that the very delicate mechanisms of the induction phenomena result from the co operation of two elements The first consists of a stimulation by a set of cells called inductive cells starting the differentiation of another set of cells A second element more important consists of the reactivity itself of the latter cells, a reactivity which expresses itself in the differentiation The alteration of one of these two elements prevents induction from being accomplished It is very easy to consider that enzymic inadequacies disturb these cellular functions in the course of deficiencies The resulting malformations are often considerable the important brain anomalies—exencephalias anophthalmias and microphthalmias—due to pantothenic acid deficiency are good examples Generally these types of malformation are precocious and *characterized by a well* defined period of embryogenesis corresponding to the various stages at which the induction phenomena occur

2 Indirect Mechanisms

The previous interpretation of the origin of the malformations produced in deficiencies suggested a direct action of the deficiencies on the embryo This assumption is confirmed by the fact that teratogenic deficiencies are observed in birds which develop isolated from maternal influence However some deficiencies can be regarded as acting indirectly through a disturbance of the maternal organism Indeed endocrinal disturbances may occur consequently altering the uterine function and the vascularization of its mucous membrane these, in their turn affecting the development of the embryo Nelson *et al* (1952 1956) *noticed that pyridoxine deficiency produces* abortion which they supposed linked to a pituitary and ovarian deficiency In any case they restored the normal state by injecting folliculin and progesterone They observed similar facts with thiamine deficiency as well as with total withdrawal of proteins (Nelson and Evans 1951 1955*a b* 1956) In their studies of vitamin E deficiencies Cheng and Bairnson (1958) also observed that the incidence of congenital abnormalities was reduced by progesterone and oestrone but the role of the excipient must be envisaged The treatment by sex hormones was without any result in the cases of pantothenic and folic acid deficiencies

XIV PRODUCTION OF TERATOGENIC EFFECTS BY SLIGHT DEFICIENCIES

What degree a deficiency must reach to have teratogenic effects is the question which now arises Hale s observations (1935) on the sow in the case of a vitamin A deficiency showed that only slight deficiencies must be involved for the mother presented no outward signs of deficiency The same thing occurred in the cow (Moore *et al* 1935) In this case Davis and Madsen (1941) added a series of chemical data—with a vitamin A content of 16 μg/100 cc of plasma no pathological symptoms were observed in the mother whilst contents between 10 and 20 μg/100 cc did not permit a correct foetal development Normal reproduction would require a content of 22–24 μg in the maternal plasma *This is a* proof that the foetus is affected much before the mother

However the occurrence of teratogenic effects with *slight vitamin A deficiencies is not* general Indeed the A deficiency which induces malformations in the rat is rather severe according to Jackson and Kinsey (1946)

As regards pantothenic acid deficiency Lefebvres (1951) has always found that the disturbances in embryonic development occur much before symptoms of deficiency in the mother The teratogenic action takes place after 14 to 20 days on a deficient diet while the first disturbances in the adult are observed only after more than a month In determinations of maternal pantothenic acid levels (Giroud *et al* 1954) in cases when embryos are resorbed *i e* in deficiencies more marked than those followed by malformations *the level was lowered only by 40 per cent at the end of* the deficiency The fact that small deficiencies have teratogenic consequences is confirmed by Lefebvres Boisselot s experiments (1955) who showed that diets still containing appreciable amounts of pantothenic acid (10–20 μg/day) quite sufficient for non pregnant females lead to malformations and resorptions

In vitamin B_2 (riboflavin) deficiency the mothers producing malformations showed no clinical sign of deficiency (Warkany 1943 Giroud 1951) Giroud

et al (1956) estimated the degree of deficiency which interfered with embryonic development by determining the riboflavin content in the liver of control rats and of rats whose litters were abnormal In the latter case the determinations showed that on the 21st day *i e* at the end of gestation the vitamin content was only one third lower than the normal value This is a rather small drop In fact the results do not really express just how small the deficiency can be and yet have teratogenic effects In these experiments in which the determinations were carried out at the end of the gestation the deficiency was maintained during the whole gestation period and was therefore continued after malformations had started The authors then studied the decrease in the riboflavin content at the teratogenic stage *i e* on the 14th day of gestation At this crucial point the animals presented a decrease in the content of riboflavin in the liver of only one fifth with respect to the controls (36 μg per gm instead of 45 μg) This shows that the development of the embryo is disturbed by a very small decrease in the state of the vitaminization of the mother (Fig 10 13)

Thus the chemical data confirm how extremely small deficiencies need be to have teratogenic effects

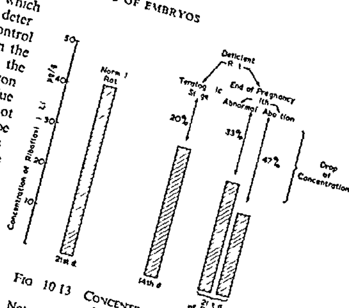


FIG 10 13 CONCENTRATION OF THE RIBOFLAVIN IN MATERNAL LIVER

Note the small drop of the vitamin content at the teratogenic stage (*Giroud et al*)

This result suggests that in the human being clinically these deficiencies can pass unnoticed in the mother

VI PRACTICAL IMPORTANCE OF DEFICIENCIES THEIR MULTIPLE CAUSES

The part played by deficiencies not only in mortality and underdevelopment but in the genesis of malformations is of considerable practical importance It is related to the ease with which these deficiencies can be produced Deficiencies especially of vitamins have many causes

1 EXOGENOUS DEFICIENCIES

The first cause of a deficiency is obviously the absence of a particular foodstuff or vitamin in the diet or its presence in too small an amount Deficiencies such as a total deficiency in only one vitamin are probably reached only in the course of laboratory experiments It is not the same with partial vitamin deficiency for an insufficient intake of a particular vitamin can easily occur This can result from an imperfect or insufficiently varied diet

The repercussions of a deficiency in essential elements necessary to a given vitamin synthesis must also be considered for without them synthesis becomes impossible Thus deficiencies of compounds which are essential precursors of vitamins can induce changes similar to or identical with deficiencies of the vitamins themselves

2 ENDOGENOUS DEFICIENCIES

A deficiency may exist owing to an insufficient endogenous production It is a well known fact that the bacteria of the digestive tract synthesize some vitamins and so are an important source of these vitamins for the organism This was well established for many vitamins of the B complex and for vitamin K If this intestinal flora becomes modified or reduced the synthesis and endogenous production of vitamins may be reduced A simple modification of the diet is enough to transform the flora and on these grounds the amount and nature of carbohydrates in food is highly important Thus Couch *et al* (1948) obtained achondroplasias in the chick embryo with diets in which the synthesis of biotin was suppressed by the use of sucrose

Very important disturbances can result from the reduction or the destruction of the normal bacteria of the intestinal tract by the use of antibiotics To produce maternal deficiencies in folic acid and in biotin antibiotics are often used such as phthalylsulphthiazole or succinylsulphathiazole Therefore repercussions from the use of antibiotics may be expected in therapeutic as well as in experimental work Di Raimondo *et al* (1952*a b*) showed that the prolonged dispensing of oxytetracycline chloramphenicol and chlortetracycline to rats strongly lowers the amount of nicotinamide folic acid cobalamine and pyridoxine in the liver The destruction of the flora with its consequences has also been observed in man after the use of intestinal or general anti-

biotics The clinical use of penicillin (Ellinger, Coulson and Benesch 1955) streptomycin (Sumner 1949) and aureomycin (Harris 1950, Leitner, 1950) demonstrated the occurrence of symptoms of vitamin B deficiencies suppressed by the administration of vitamins Di Raimondo *et al* (1952*a*, *b*) have studied in man the administration of oxytetracycline chlortetracycline and chloramphenicol which is followed by a marked reduction of the vitamin urinary excretion parallel with the disappearance of intestinal bacteria especially of the Coli type Harris (1950) considered that a coated tongue, a vaginitis and a vulvitis after a chlortetracycline treatment were due to a vitamin B_2 deficiency Tomaszewski (1953) after various antibiotics and Hartmann and Stahel (1950) after penicillin noticed pellagrous signs curable by nicotinic acid Merliss and Hoffmann (1951) considered that steatorrhea after antibiotics would result from a deficiency itself due to the destruction of the flora Sacks *et al* (1951) described anaemias due to a folic deficiency after streptomycin treatment Lattes *et al* (1952) described mouth lesions after terramycin Lhermitte (1952) noted similar facts and Planson (1951) insisted on their prophylaxis by the B complex Some antibiotics can also disturb the production of vitamin K (Humphrey *et al* (1953)

All these facts which prove that vitamin deficiencies may result from the use of various antibiotics show that accidental deficiency can occur during gestation following an antibiotic treatment of some infection Filippi and Mela (1957) gave tetracycline to rats from the 5th to the 10th day of gestation They obtained offspring with cleft palates shortened limbs and syndactylia *i e* malformations observed in the case of a riboflavin deficiency These accidents were suppressed if the B complex was given simultaneously Here is a good example of a teratogenic vitamin deficiency due to the destruction of the intestinal flora

3 Elimination

On the other hand a deficiency can result from the occasional elimination of vitamins For instance the dispensing of powdered charcoal causes the adsorption of some B vitamins in the intestine resulting in a secondary deficiency in these vitamins (Cailleau and Adrian 1948) The administration of paraffin oil which dissolves and carries away vitamin K can produce a deficiency in this vitamin (Elliot 1940)

4 Defective Utilization

A state of deficiency can result from an insufficient utilization of an available vitamin that is from its inability to be converted into an active element *i e* into an enzyme According to Hoet (1956) a lack of vitamin A could result from a metabolic disturbance in diabetic states He noticed in diabetic and prediabetic pregnant women an abnormal carotenaemia and tendencies to foot sole keratosis and above all a hemeralopia all the symptoms of which indicated that the alimentary carotene was unable to be transformed into vitamin A The transformation of carotene into vitamin A is inhibited in cases of alloxanic diabetes in the rat (Sobel *et al* 1953) Clark and Colburn (1955) also noticed that cortisone prevents this transformation

5 Antagonists

A deficiency can result from the failure of a vitamin or other substance to become attached at the point where it must be present or active this is what happens if a substance of similar chemical structure (analogue antagonist) is substituted the substance is then unable to function Galactoflavin thus interferes with the utilization of lactoflavin and pantoyltaurine and ω methylpantothenic acid have the same effect with respect to pantothenic acid similarly methylfolic acid and aminopterin affect folic acid function

These effects which are mainly experimental are not limited to vitamins since amino acids may also be affected by antagonists

6 Toxins

Vitamin disturbances and deficiencies can result from the action of some toxins Landauer s observations (1953) are highly suggestive in this respect Micromelia produced by treatments with insulin sulphanilamide and eserine can under certain conditions be completely prevented by the administration of nicotinamide The malformations induced by boric acid are similar to those of riboflavin deficiency the liver of the treated animals having a riboflavin content markedly lower Boric acid would form with this vitamin a complex in which form the vitamin is unavailable

7 Unbalanced Diets

Finally, some deficiencies have quite different causes for example, diets which are not well balanced

The addition of a particular element to a well constituted diet and therefore one containing all vitamins in suitable amounts creates an unbalanced state—disturbances appear corresponding to a deficiency in other elements These deficiencies were called secondary deficiencies by Randoin (1951) and deficiencies without deficiencies by Bisceglie (1949) Facts of this kind were observed during nutritional researches on reproduction Lepkovsky *et al* (1938) observed during their experiments that the addition of vitamin B_1 to certain diets decreased the number of hatched eggs Ross *et al* (1944) noted

that supplements of riboflavin and choline increased the incidence of malformations in piglets whose mothers were fed an already deficient diet In our laboratory Lefebvres (1951) produced particularly striking results in experiments on pantothenic acid deficiency By adding large amounts of nicotinamide to the diet the deficiency became more severe—instead of 8 normal young per litter there were 4–5 abnormal embryos 3 resorbed and only 1 normal embryo per litter Neuweiler (1955) and Mouriquand *et al* (1935) noticed gestation troubles after an excess of vitamin B_1 or vitamin C Cohlan (1953) and Giroud and Martinet (1955) also obtained many abnormal embryos by means of a large excess of vitamin A but these experiments were carried out rather under toxicologic conditions than under nutritional ones

We can therefore conclude that vitamin deficiencies have various causes that these deficient states can easily arise and consequently occur much more frequently than is usually appreciated

XVI THE ROLE OF THE GENETIC CONDITIONS

The individual responses of mothers and embryos to deficiencies raises the question of the genetic factors involved even when experiments are carried out with well-defined strains when working in this field it might be worth making a study of twins produced for example by the Tatu (Dasypus) However if we pass from one race or strain to another new genetic factors may come into play disturbing the development more easily

With a diet deficient in vitamin A Andersen (1941) observed 0 9 per cent of diaphragmatic hernias in rats of Long Evans strain a strain which produces no spontaneous hernia On the other hand he obtained 19 per cent with the same deficient diet in the strain DH in which the spontaneous incidence is 2 7 per cent with a normal diet In the first case the deficiency causes some malformations in the second case it causes many more for the morphogenetic mechanism is more easily disturbed in this strain Kalter and Warkany (1957) have observed very different results in riboflavin-deficient mice according to the strain cleft palate 2 8 per cent in A strain and 41 per cent in DBA syndactylia 4 8 per cent in DBA and 82 3 per cent in 129 strain

It is likely that similar genetic influences are often at play and it can be assumed that a deficiency which proved teratogenic in a certain strain is not necessarily so in another In man the same will happen when passing from one individual to another This influence of the hereditary patrimony is found in all experiments on teratogenesis It was especially detected during anoxias (Ingalls *et al* 1950) as well as in teratogenesis by toxins Thus Landauer (1953) noticed important variations in the teratogenic effects of boric acid in different strains of hens these variations might have been related to the various riboflavin requirements of these strains

A fortiori these questions arise when we pass from one species to another Nevertheless teratogenic sensitivity must exist everywhere even if it is somewhat varied

XVII TERATOGENESIS IN VARIOUS SPECIES

The numerous deficiency experiments that we have reported point to the conclusion that teratogenic action is found in a number of species belonging to distinct groups though with some variations Indeed it was observed in birds such as chicken turkey duck and in mammals as different as pig cow rat mouse rabbit cat We may therefore wonder whether it would not be met with in all our domestic species and even in the human race if only we looked for it (Plate IV (*f*) and (*g*))

Houet and Lecomte Ramioul (1950) attributed a total hare lip in an infant to a maternal B_2 deficiency however the teratogenic role of this vitamin deficiency was not confirmed by Braun *et al* (1945) They studied in Palestine 900 pregnant women among whom 190 showed symptoms of riboflavin deficiency in the second part of their pregnancy symptoms which quickly disappeared after delivery They noted only premature births foetal deaths and disturbance of pregnancy but no congenital anomalies However it is a well known fact that a deficiency detected at the end of gestation does not necessarily justify the inference of the existence of a deficiency during the teratogenic stages

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On the other hand it is nearly certain that a deficiency in folic acid is teratogenic in human beings This is shown by Thiersch s observations (1952) he treated pregnant women with aminopterin an anti vitamin of folic acid in order to produce abortions In several cases he noticed anomalies in 3 foetuses out of 10 such an incidence cannot be due to chance In a recent series of treatments Thiersch (1956) observed a case of anencephaly The observation of other similar facts may be expected in man According to Hoet *et al* (1955) the malformations which are so frequently noticed in the foetuses of diabetic or

prediabetic women may in fact result from a deficiency in vitamin A

A general difficulty in this field resides in the individual requirements which we have already mentioned However, the main obstacle to the clinical detection of a teratogenic deficiency in humans lies in the fact that it is only very slight and therefore easily passes unnoticed

XVIII CONCLUSIONS

The nutritional needs of the embryo are important They increase in absolute value with age *They must* be satisfied General deficiencies do not have as bad results as specific deficiencies (vitamins, amino acids some unsaturated fatty acids)

If there are nutritional deficiencies, some of their repercussions on the embryo or the foetus may be more or less similar to those on any organism, these deficiencies can retard growth, produce lesions and even cause death But they can have also very particular repercussions, specific to the stage of development, they can induce malformations At a definite *time early in development* the embryo is the subject of particular mechanisms of morphogenesis and if these are disturbed malformations occur Their appearance is therefore characteristic of nutritional disturbances at this stage especially of vitamin deficiencies

From all these points we can draw the general conclusion that the proper nourishment of the maternal organism is of very great importance to the production of healthy, properly formed young

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